FIXED-FILM ANAEROBIC DIGESTION: MECHANISMS OF PATHOGEN REDUCTION AND IMPACTS ON VIRUS ADSORPTION TO SOIL

By

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To Ethan and Joshua.

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Anaerobic digesters have been used for treatment of various wastewaters. Systems are usually operated at mesophilic (35°C) temperatures and long hydraulic retention times (HRTs) (> 20 days). Pathogen reduction during anaerobic digestion is mainly correlated with operating temperature and HRT. Higher temperatures and longer HRTs result in increased rates of pathogen decimation.

Previous studies show that a novel fixed-film anaerobic digester, operated at a low HRT (≤ 3 days) and ambient temperature (≤ 28°C) treating flushed dairy manure wastewater, achieved significant reductions of indicator and pathogenic bacteria. Thus, we sought to determine factors that contributed to indicator and pathogenic bacteria reduction during operation at a low HRT and ambient temperature. The presence of indigenous microflora was found to reduce the proliferation of indicator and pathogenic bacteria. Bacteriophages were also inactivated by the presence of indigenous microflora. Also, decimation of *Staphylococcus aureus* was attributed to starvation, resulting from

anaerobic digestion, while not suppressing the other test organisms to the same degree. Furthermore, attachment to the retained biofilm (i.e., fixed-film) contributes to reduction by removing indicator and pathogenic bacteria from the liquid phase during anaerobic digestion.

Next, our study examined the impact of anaerobic digestion on virus adsorption to soil following land application of treated wastewater. Anaerobic digestion increased retention of viruses to the soil and decreased mobility of attached viruses through the soil matrix, as compared with untreated wastewater. Anaerobic digestion removed compound(s) that interfered with virus adsorption to soil. Initial characterization showed that these compound(s) were less than 100 kDa in size. These compound(s) interfered with hydrophobic and electrostatic interactions used by the viruses for adsorption to soil. Futhermore, compound(s) less than 10 kDa in size caused inactivation of MS2.

Our results show the critical role of indigenous microflora and the retained biofilm during anaerobic digestion in a fixed-film system. The indigenous microflora and attachment to the biofilm causes decimation of indicator and pathogenic organisms from flushed dairy manure wastewater, thereby reducing the environmental load of these organisms during land application. Anaerobic digestion increases the retention of residual viruses within the soil matrix, therefore reducing the likelihood of contaminating groundwater.

CHAPTER 1
INTRODUCTION

Livestock are known reservoirs of pathogenic organisms and these organisms are shed with feces. Also, animal husbandry generates over 1.6 billion tons of manure per year in the United States (Altekruse et al., 1997). Pathogenic organisms in manure may be introduced to the environment by land application. Application of solid manure (as fertilizer) or diluted manure slurry (for fertirrigation) to land is a common management strategy used by concentrated animal feeding operations (CAFOs). Although this practice provides nutrients for crop production, pathogenic organisms may persist in manure, and potentially introduced to the environment and eventually to the herd and public (Hill, 2003).

In regards to public health, animal manure is a source of food contamination and has been linked to outbreaks of foodborne illnesses (Park and Diez-Gonzalez, 2003). Food can become contaminated with pathogenic organisms associated with manure during food processing (i.e., slaughter) or by land application (i.e., contamination of uncooked fresh produce) (Troutt et al., 2001). Therefore, pathogenic organisms introduced by manure into the food chain can result in outbreaks of foodborne illnesses.

Although viruses and protozoa may also be associated with foodborne illnesses, bacteria have been found to be the most common cause of outbreaks in the United States. The Centers for Disease Control and Prevention (CDC) reported 75% (655 of 878) of foodborne-disease outbreaks from 1993 to 1997 were of bacterial origin (Centers for Disease Control and Prevention, 2000). Furthermore, the Economic Research Service

(ERS) of the United States Department of Agriculture estimated the annual cost associated with five bacterial agents of foodborne illnesses to be $6.9 billion.

Effective treatment strategies must be used to reduce the levels of pathogenic bacteria in manure. However, to provide an incentive to implement new technology on CAFOs, treatment strategies should not only reduce the level of pathogenic organisms, but also offer on-farm and environmental benefits. Therefore, anaerobic digestion is a treatment strategy that can be used to reduce pathogen levels in manure and offer on-farm benefits such as bioenergy production, odor reduction, and biofertilizer production. A fixed-film anaerobic system at the University of Florida Dairy Research Unit (DRU) has been shown to decrease pathogen levels and supply bioenergy and biofertilizer for on-farm use (Davis et al., 2001, Wilkie, 2000)

The persistence of pathogenic organisms in manure can potentially result in public health concerns. Current manure management strategies used by CAFOs may be improved to control the levels of pathogenic bacteria in manure. Improvements should include implementing a manure treatment technology that offers on-farm and environmental benefits in addition to controlling pathogenic bacteria associated with manure. Thus, anaerobic digestion is a viable option that offers pathogen control and on-farm benefits. The fixed-film anaerobic system used by the DRU has been shown to offer on-farm benefits while reducing the level of pathogens from manure. However, the factor(s) that contribute to pathogen reduction during fixed-film anaerobic digestion and the effect on pathogen transport through soil following land application of treated wastewater remains unknown. Therefore the purpose of the current study was to: 1) determine the factor(s) that contribute to pathogen reduction during fixed-film anaerobic

digestion and 2) determine the effect of fixed-film anaerobic digestion on virus transport through soil after land application.

CHAPTER 2
PATHOGENIC ORGANISMS IN BOVINE MANURE AND THEIR REDUCTION BY ANAEROBIC DIGESTION

Pathogenic bacteria that pose herd and human health concerns are associated with manure (Bicudo and Goyal, 2003, Huston et al., 2002, Wang et al., 1996, Wells et al, 1991). Furthermore, pathogenic bacteria can survive in manure for several days once voided from the animal (Himathongkham et al., 1999). Therefore, exposure to manure can potentially cause animal diseases, resulting in lost revenues for the animal operation. Also, environmental contamination may result in human exposure to zoonotic organisms, which may lead to potential diseases. Environmental contamination and transmission of zoonotic organisms to humans from livestock manure has been the focus of recent legislation to improve manure handling and treatment strategies (U.S. Environmental Protection Agency, 2003). Another concern with manure is the emergence of antibiotic resistant strains of pathogenic bacteria. The emergence of antibiotic resistant pathogenic bacteria from manure of antibiotic-fed animals can pose severe herd and human health issues (Dargatz, 1998). Therefore, the threat of antibiotic resistant pathogenic bacteria requires that better and more effective pathogen reducing manure management strategies be implemented by animal operations.

Pathogenic Organisms in Dairy Cattle Manure

Several types of pathogenic bacteria have been isolated from animal manure (Pell, 1997): *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, and *Mycobacterium paratuberculosis*. Pathogenic viruses and protozoan have also been isolated from animal

manure (Chang et al., 1997, Olson et al., 2004, Valle et al., 1999). Some organisms are shed in high concentrations when the animal displays illness or may be shed in feces where the animal does not exhibit any clinical signs of disease. In either case, manure is a source of pathogenic organisms that may cause either human or herd diseases.

Zoonotic Bacteria

***Escherichia coli* O157:H7**. *Escherichia coli* O157:H7 is an enterohemorrhagic strain of *E. coli*, which produces a Shiga-like toxin known as verocytotoxin. The verocytotoxin is a causative agent of several diseases such as, hemolytic uremic syndrome (HUS), thrombocytopenic purpura, and hemorrhagic colitis (Pell, 1997, Prescott et al., 1999, Wells et al., 1991). This organism is considered an emerging pathogen and has been isolated from raw beef and milk products (Callaway et al., 2003).

Dairy cattle serve as a reservoir of *E. coli* O157:H7, where younger animals harbor the organism more so than mature animals. Wells et al. (1991) measured *E. coli* O157:H7 concentrations in fecal samples from cattle in herds associated with two sporadic cases of hemolytic uremic syndrome and an outbreak of gastroenteritis. The investigation revealed that 2.3% of calves (5 of 210), 3.0% of heifers (12 of 394), and 0.15% of adult cows (1 of 662) shed *E. coli* O157:H7 in feces. Doyle et al. (1997) also found that *E. coli* O157:H7 is isolated predominantly from young animals, with the highest rate of isolation from postweaned calves. Zhao et al. (1995) found that *E. coli* O157:H7 was shed more frequently by weaned calves (4.9 to 5.3%) than preweaned calves (1.5 to 2.9%). These results show that not only are dairy cattle reservoirs of *E. coli* O157:H7, but younger animals tend to harbor the organism more so than adult animals.

Escherichia coli O157:H7 can not only be shed in feces, but also proliferate and persist in feces once introduced into the environment. Wang et al. (1996) showed that *E. coli* O157:H7 could survive and proliferate in bovine feces up to 21 days at 37°C, 49 to 56 days at 22°C, and 63 to 70 days at 5°C, although no growth occurred at 5°C. Nicholson et al. (2005) found that *E. coli* O157:H7 could survive for 93 days in dairy manure slurry stored at less than 20°C. However, survival decreased to 32 days following land application without storage. Park and Diez-Gonzalez (2003) found that *E. coli* O157:H7 could survive in cattle manure for at least 25 days at room temperature.

***Salmonella* spp.** *Salmonella* spp. is the most common pathogen associated with livestock manure (Stehman, et al., 1996). These organisms are facultative anaerobic, gram-negative rods. These organisms are non-species specific (e.g., equine, swine, poultry, and bovine), can be transferred from animals to humans through undercooked meat or raw eggs, and are causative agents of typhoid fever, enteric fevers, septicemia, and gastroenteritis. *Salmonella* spp. causes gastroenteritis in humans and can be fatal to young, elderly, or immuno-compromised individuals (Dargatz, 1998). From 1993 to 1997, 55% of bacterial foodborne-disease outbreaks in the United States were caused by *Salmonella* spp (Centers for Disease Control and Prevention, 2000). The Economic Research Service (ERS) estimated annual economic loss due to foodborne *Salmonella* infections in 1996 and 2003 was $3.5 and $2.9 billion, respectively (Buzby et al., 1996, ERS, 2003, Frenzen et al., 1999).

The emergence of antimicrobial resistant *Salmonella* spp. has become a major health concern. In particular, *S. typhimurium* DT104 has developed resistance to various antibiotics includeing ampicillin, sulfonamides, tetracycline, chloramphenicol, and

streptomycin (Dargatz, 1998). Park and Diez-Gonzalez (2003) found that *S. typhimurium* DT104 could survive in cattle manure for at least 25 days at room temperature. During slaughter, meat can potentially become contaminated with *S. typhimurium* DT104. Galland et al. (2001) identified 59 isolates as *S. typhimurium* in a survey of five slaughter establishments processing cull (market) dairy cattle. Out of the 59 isolates, 35 were obtained from one facility, where 88.6% of those isolates were confirmed as *S. typhimurium* DT104.

Salmonella spp. are commonly associated with dairy cattle. Huston et al. (2002) found that out of 2,283 individual fecal samples and 15 bulk milk tank samples from five dairy herds, 11% of calves, 56% of mature cows, and 20% of bulk tank samples were positive for *Salmonella* spp. The study also found that mature cows were 21 times more likely to be shedding *Salmonella* spp. than were unweaned calves. Furthemore, no clinical signs of salmonellosis were identified in any of the adult animals in the five herds. The study concluded that *Salmonella* spp. can be shed in feces of mature healthy cows, however bacterial concentrations were not determined during the study.

Dairy cattle can also serve as a vehicle for *Salmonella* spp. contamination of meat. Troutt et al. (2001) measured prevalence of *Salmonella* spp. in five beef slaughter establishments in five different regions of the United States. The investigators found a 23.1% overall prevalence of *Salmonella* spp. Additionally, the highest prevalence (54.5%) and the lowest (4.3%) prevalence were found during the summer sampling period and in two different locations. The investigators were able to detect *Salmonella* spp. in all the establishments surveyed. These results suggest that the prevalence of *Salmonella* spp. is not dependant on seasonal variations nor is the organism isolated to

one region of the country. Galland et al. (2001) determined *Salmonella* serotype diversity and prevalence from the same samples obtained in the study by Troutt et al. (2001). The investigators identified 58 serotypes of *Salmonella*, where *Salmonella* ser. Montevideo was the most frequent (21%). Other serotypes (Muenster, Kentucky, Mbandaka, Senftenberg, and Typhimurium) were found in all establishments surveyed.

***Campylobacter* spp.** *Campylobacter* spp. are microaerophilic, gram-negative vibriod cells. They are usually found in the oral cavity, reproductive organs, and intestinal tract of animals and humans. Two species of particular concern are *C. jejuni* and *C. coli*. These organisms cause human enteritis and are associated with livestock. *Campylobacter* spp. may enter the supply of raw milk or water from bovine feces (Wesley et al., 2000). Healthy cattle are potential reservoirs of *C. jejuni* and *C. coli* and the incidence of disease is seasonal (Wesley et al., 2000).

Mycobacterium paratuberculosis. *Mycobacterium paratuberculosis* is an aerobic, acid-fast, rod-shaped organism. This slow-growing, fastidious organism generally requires the presence of mycobactin, a growth factor that serves as an iron chelator, to proliferate (Grant et al., 1996). *Mycobacterium paratuberculosis* is the causative agent for Paratuberculosis (PTB), or Johne's disease, in cattle and has been implicated in Crohn's disease in humans (Grant et al., 1996). Paratuberculosis is a chronic, granulomatous intestinal disease that is manifested by nonresponsive diarrhea, progressive weight loss, and death. *Mycobacterium paratuberculosis* infections are not confined to specific tissues and the organism can be isolated from several organs (Hines et al., 1987). This organism has also been isolated from uterine flush fluids of infected dairy cattle (Rohde et al., 1990).

Several techniques have been developed to detect *M. paratuberculosis*. Collins et al. (1990) described the use of radiometry for detection. A study of nine infected dairy herds in Wisconsin showed that radiometric detection using filter-concentrated manure samples detected 92% of *M. paratuberculosis* from feces, while conventional culture techniques using HEY agar only detected 60%. Molecular detection of *M. paratuberculosis* has also been studied. Millar et al. (1996) found that IS900, an unusual DNA insertion element unique to *M. paratuberculosis*, can be used to detect this organism in pasteurized milk.

Paratuberculosis has an adverse effect on milk production (Hernandez and Baca, 1998). *Mycobacterium paratuberculosis* can be shed in milk in high concentrations (Sweeney et al., 1992) and can survive pasteurization temperatures (Grant et al., 1996). This organism can also be transmitted in utero (Seitz et al., 1989) and by herd-to-herd contact (Collins et al., 1994). The prevalence of this organism at a dairy can be attributed to environmental conditions, manure-handling practices, newborn calf care, and grower calf care (Goodger et al., 1996).

Listeria monocytogenes. *Listeria monocytogenes* are facultative anaerobic, gram-positive, rod-shaped organisms. These organisms cause listeriosis in animals and humans (George et al., 1996, Jensen et al., 1996, Mussa, et al., 1999). Jensen et al. (1996) found that *L. monocytogenes* isolated from humans and cows were of the same serotype. This implies that milk contaminated with this organism can possibly infect humans.

The ability of this organism to survive in food causes concern for human health (George et al., 1996). Gohil et al. (1996) found that *L. monocytogenes* can survive up to three days in labneh and houmos, two traditional Arabic foods. *Listeria monocytogenes*

have also been shown to survive in acidic foods (e.g., yogurt, cottage, mozzarella, and cheddar cheeses) by induction of the acid tolerance response (ATR) (Gahan et al., 1996). The ATR mechanism involves the synthesis of proteins, including outer membrane and heat shock proteins, which provide a mechanism for maintaining intracellular homeostasis in suboptimal, or lethal, environments.

Another concern for human health is that antibiotic resistant strains of these organisms have been detected. Erythromycin resistant (Em^r) and tetracycline resistant (Tet^r) *Listeria* spp. has been isolated from food (Roberts et al., 1996). The use of high pressure (600 MPa) has been studied as a possible treatment process for food. Mussa et al. (1999) found that the use of high pressure in the pasteurization reduced the concentration of *L. monocytogenes* in milk.

Mastitic Bacteria

Staphylococcus aureus. *Staphylococcus aureus* are gram-positive, facultative anaerobic cocci. These organisms are common inhabitants of the skin and mucous membranes of warm-blooded animals. *Staphylococcus aureus* are causative agents of peracute, acute, chronic, subclinical mastitis in dairy cattle and can be excreted in milk (Umeki et al., 1993). Subclinical infections are the most common form of mastitis caused by *S. aureus*, however this organism can also cause clinical mastitis (Elbers et al., 1998). *Staphylococcus aureus* induced mastitis (i.e., contagious mastitis) is characterized by invasion of the mammary gland tissues, where the mammary gland parenchyma is replaced by granulation tissue, fibrosis occurs, and multiple abscesses form (Cullor, 1993). Although antibiotic therapy has been used to treat infections, resistant strains of *S. aureus* associated with mastitis have emerged in recent years (Malinowski and Klossowska, 2003).

***Streptococcus* spp.** Streptococci are facultatively anaerobic gram-positive cocci. These organisms have a fermentative metabolism where lactate is the primary product. These organisms are common inhabitants of the mouth and upper respiratory tract of vertebrates. Several species of streptococci are causative agents of bovine mastitis. A streptococcus responsible for contagious mastitis is *S. agalactiae. Streptococcus uberis* and *S. dysgalactia* are associated with environmental mastitis.

Contagious mastitis infections caused by *S. agalactiae* (Lancefield group B) can be either clinical or subclinical. Infections involve adherence of the organism to mammary gland cells. Although, Lammers et al. (2001) showed poor adhesion to mammary gland cells by *S. agalactiae,* fibrinogen-binding proteins secreted by the organism have been shown to aid in adhesion to mammary gland cells (Jacobsson, 2003).

A single strain of *S. agalactiae* has been shown to be the causative agent of mastitis. Merl et al. (2003) studied 79 streptococcal isolates from subclinical mastitis of 54 cows. The study found that a single strain of *S. agalactiae* were responsible for all the infections. In addition to mastitis, *S. agalactiae* can cause severe invasive disease in humans, especially in neonates (Bohnsack et al., 2004). Evidence suggests that *S. agalactiae* may be a zoonose, being transmitted from cows to humans and visa versa, although the risk of transmission between species is considered low (Sukhnanand et al., 2005).

Environmental mastitis infections may be related to environmental factors such as insect population (Yeoman and Warren, 1984) and bedding materials (Hogan et al., 1990). "Summer mastitis" is a teat infection caused by several organisms including *S. dysgalactiae* and is spread by a fly, *Hydrotaea irritans* (Yeoman and Warren, 1984).

Summer mastitis infections caused by *S. dysgalactiae* were found to be five times more prevalent in herds without fly control versus herds with fly control (Nickerson et al., 1995).

The type of material used for bedding can result in increased herd exposure to environmental mastitic streptococci. Care and type of bedding material (i.e., organic or inorganic) used in freestall barns have a direct impact on exposure to environmental mastitic streptococci (Bey et al., 2002). Hogan et al. (1990) found that environmental mastitic streptococci were able to proliferate in organic bedding material and increase the concentration of these organisms on teats. Zehner et al. (1986) found *S. uberis* was able to proliferate in various types of organic bedding (e.g., recycled manure, chopped straw, hardwood chips, paper, and softwood sawdust). Additionally, Todhunter et al. (1995) reported streptococci concentrations of 6 to 7 $\log_{10}$ CFU/g dry weight of various bedding materials (i.e., pelleted corn cobs, recycled manure, and wood shavings) throughout the year. Although streptococci concentrations did not vary with seasonal conditions, the rate of environmental streptococcal mastitis (reported as intramammary infections per cow per day) was highest during the summer and lowest during the winter. These results suggest that the use of organic bedding material can lead to environmental mastitis by providing conditions for mastitic streptococci to survive and proliferate.

Pathogenic Viruses

BVDV. Bovine virus diarrhea is an infectious disease in cattle caused by a retrovirus, BVDV. BVDV is a member of the *Pestivirus* genus, within the Flaviviridae family. The attachment and entry of flavivirus is mediated by the envelope (E) protein (~50 kDa), a major glycoprotein on the virus particle (Lee and Lobigs, 2000). BVDV is found in oculonasal discharges and is transmitted by close and direct contact within the

herd. An important feature of this disease is that an infected pregnant female can give birth to a persistently infected calf that will excrete BVDV throughout its life (Valle et al., 1999). Valle et al. (2000) studied BVDV sero-conversion (a surrogate measure for incidence) in Norwegian dairy herds and found that there was a steady decline in sero-conversion risk. Although sero-conversion continued over time, the decrease was attributed to effective control of major risk factors. These factors include the use of common pastures, purchasing animals associated with BVDV, and herd-to-herd contact over pasture fences (Valle et al., 1999).

Bovine rotavirus. Bovine rotavirus is a member of the Reoviridae family, has a genome that consists of 11 segments of dsRNA, and is enclosed by a triple-layered capsid. Rotaviruses are a common cause of diarrhea in calves and humans (Chang et al., 1997, Klingenberg et al., 1999). There are three groups of rotaviruses: A, B, and C. Group A is a common cause of neonatal calf enteritis (Klingenberg et al., 1999). Group B has been implicated in sporadic cases of diarrhea in calves (Chang et al., 1997). Group C has been suggested as a common enteric pathogen in animals and humans, but are difficult to cultivate because they are fastidious in their in vitro cell culture requirements (Alfieri et al., 1999).

Activation of rotavirus infectivity requires the presence of exogenous proteases, which cleave the VP4 protein. Replication is completely cytoplasmic, where rotaviruses supply necessary enzymes to replicate dsRNA. Subviral particles form and maturation occurs through budding of the endoplasmic reticulum (ER). Finally, viral particles are released through cell lysis.

Two major proteins, VP4 and VP7, on the outer shell of rotaviruses have been studied extensively (Alfieri et al., 1999, Chang et al., 1997, Klingenberg et al., 1999). The genes of these two outer capsid proteins are used for molecular detection of rotaviruses using reverse transcription polymerase chain reaction (RT-PCR). Buesa et al. (1996) found that RT-PCR is highly effective for G (VP7 gene) and P (VP4 gene) genotyping of rotaviruses, but not substantially more sensitive than enzyme linked immunosorbant assay (ELISA) and electron microscopy (EM) for detection in fecal and environmental samples. To control the spread of this pathogen, preventive measures should be aimed at better hygiene, herd management, and vaccination programs (Klingenberg et al., 1999).

Parasitic Protozoa

Cryptosporidia. Cryptosporidia are protozoan parasites that are commonly associated with human and livestock manure (Olson et al., 2004). The organism exists as an oocyst during the infectious stage. Oocyst are biologically dormant, easily transported in water and can survive drinking water treatment (i.e., chlorination). Although there are several species of cyptosporidia (e.g., *C. andersoni* and *C. muris*), only *C. parvum* is clinically significant. *Cryptosporidium parvum* is the causative agent of cryptosporidiosis in humans, which is characterized by severe diarrhea and dehydration.

Cattle are considered the major reservoir of *C. parvum* (Olson et al., 2004). Younger calves are more susceptible to infection and are more likely to shed oocysts during infection than are older animals. Sischo et al. (2000) measured the prevalence of cryptosporidia in dairy cattle and calves. The investigators detected *Cryptosporidium* in 10 of 11 farms surveyed. Furthermore, calves (0 to 3 weeks old) had the highest prevalence of cryptosporidia infections among older calves ($\geq$ 4 weeks old) and mature

cows. Additionally, the investigators evaluated several risk factors for spreading crytosporidia among calves. Contact between calves and frequent bedding changes (> 12 times per year) were found to be the two main management risk factors associated with fecal shedding of cryptosporida. Younger calves are more likely to become infected and shed oocysts. However, adult animals are can shed oocysts where infections are asymptomatic (Graczyk et al., 2000).

The field spreading of contaminated calf manure can potentially introduce oocysts to the watersheds (Walker and Stedinger 1999). To contaminate watersheds, oocysts must transported by manure and released into a watershed. Schijven et al. (2004) measured oocyst release from manure by water application either as a mist or droplets. Calf and cow manure were used separately or mixed together in the study to simulate manure management practices at the dairy operation. The study found that oocysts were more readily released from calf manure than cow manure. The larger coarse particles (i.e., undigested hay or grain) in cow manure do not readily partition into the aqueous phase. However, calf manure consisted of finer colloidal particles, which partitioned easily into the aqueous phase. The differences in manure composition are directly impacted by the difference in diet among cows and calves. The study also found that droplet size and water application rate was directly proportional to oocyst release rates. This result suggests that during heavy rainfall events, oocysts can be released from manure and potentially be transported in runoff.

Anaerobic Digestion and Pathogenic Organisms

Anaerobic Digestion

Anaerobic digestion is the natural process of converting complex organic compounds to methane and carbon dioxide by a series of symbiotic microbial interactions

in the absence of oxygen. The process of anaerobic digestion is utilized as a means of degrading organic matter in the environment, providing nutrition for ruminants, and treating different industrial and agricultural organic waste products. Ruminant organisms have evolved to utilize anaerobic digestion for degrading and deriving energy from otherwise indigestible plant material. Anaerobic degradation under controlled conditions provides an effective method for treating organic wastes. The development of enclosed systems (i.e., anaerobic digesters) has enhanced the process of anaerobic digestion by providing an optimum environment for methanogenesis.

Anaerobic Digesters

Anaerobic digesters are bioreactors designed to optimize the process of anaerobic degradation by providing an environment under controlled conditions to facilitate the growth of the anaerobic bacteria. These systems are designed for wastewater stabilization and energy production (i.e., methanogenesis). Other benefits derived from anaerobic digesters are odor reduction (Powers, 1999) and bacterial pathogen decimation (Kearney, 1993). Several types of digesters have been studied for treating manure. These include anaerobic lagoons, continuous stirred tank reactors, plug-flow digesters, and fixed-bed reactors.

Anaerobic Lagoons. Preceding digesters, anaerobic lagoons have been used to treat animal manures. However, lagoons do not offer biogas recovery for energy production. Development of covers over lagoon systems permitted biogas recovery for energy use. These systems are operated at ambient temperature, resulting in seasonal changes in biogas production. Covered anaerobic lagoons require hydraulic retention times (HRTs) in excess of 40 days. These systems have been found to achieve COD reductions of 70% to 94% and produce biogas with various methane contents (70% to

88% CH_4) (Cheng et al., 1999, Safley and Westerman, 1992, Williams and Frederick, 2001).

Continuous Stirred Tank Reactors. Continuous stirred tank reactors (CSTRs) are insulated, cylindrical tanks that are either continuously or intermittently fed, where the volume of effluent equals the volume of influent. These systems are used for methane recovery from livestock manure that usually contains a 5% to 12% total solids concentration. The contents of CSTRs are mixed either by mechanical methods or recycling biogas through the liquid. These systems are operated at HRTs of 10 to 20 days (Wilkie and Colleran, 1989) and maintained at either mesophilic (~35°C) or thermophilic (~55°C) temperatures, although psychrophilic (5°C to 20°C) operating temperatures have been studied (Zeeman et al., 1988).

Plug-flow Digesters. Plug-flow digesters are systems used to treat scraped manure with an 11% to 14% total solids concentration (Hills, 1983, Hills and Mehlschau, 1984, Liu, 1998). They are usually long rectangular tanks, often built into the ground, and equipped with an impermeable plastic cover for biogas recovery. Manure is not mixed as it moves through the system as a combined mass or "plug". Plug flow systems usually operate at HRTs in excess of 30 days.

Fixed-bed Reactors. Fixed-bed anaerobic reactors use a support medium to retain anaerobic organisms. A fixed-bed system is constructed with an internal support media to provide surface area for the attachment and retention of anaerobic bacteria as a biofilm. This advancement allows for the retention of anaerobic bacteria within the digester independent of HRT (Wilkie, 2000). Adhesion of anaerobic bacteria to inert support media, facilitating biofilm formation, is influenced by bacterial surface properties

and support media characteristics (Verrier et al., 1987). The surface texture and porosity of the support media have a significant impact on system performance, where open-pored surface texture and high porosity results in higher biofilm efficiency (Show, 1999). A major advantage of fixed-bed technology is the immobilization of methanogens, which grow at a slow rate (Wilkie and Colleran, 1989). Fixed-bed technology allows for the bacteria to be retained within the digester, resulting in a decrease in HRT needed to digest wastewater (Wilkie, 2000). This type of wastewater management system is capable of treating larger volumes of dilute wastewater per unit time than conventional systems (Wilkie, 2000). Fixed-bed anaerobic reactors are suited for treating slurry with low (< 1%) total solids concentrations.

Microbial Diversity in Anaerobic Digesters

The microbial ecosystem within anaerobic digesters is primarily a consortium of bacteria that are involved in synergistic interactions for the digestion of organic compounds for biogas production (Bitton, 1994, Wilkie and Colleran, 1987). The process of digestion, involves four groups of bacteria: 1) hydrolytic bacteria, 2) acidogenic bacteria, 3) acetogenic bacteria, and 4) methanogenic bacteria. The four groups of bacteria are dependent on each other for maintaining an optimum environment for anaerobic digestion.

Hydrolytic Bacteria. Hydrolytic bacteria degrade complex organic material (e.g., polysaccharides, proteins, lipids), yielding monomers that are subsequently utilized by the acidogenic bacteria. Hydrolytic species inhabiting digesters feed ruminant manure originate from organisms that survive passage from the rumen and into feces. Velázquez et al. (2004) identified a novel species of *Paenibacillus, P. favisporus* sp. nov. This

organism was isolated from bovine feces and found to produce a variety of hydrolyic enzymes, including xylanases, cellulases, and amylases.

Acidogenic Bacteria. The acidogenic bacteria convert the monomers (e.g., monosaccharides, fatty acids, amino acids) to organic acids, alcohols, and ketones. Acidogenic bacteria include *Propionium* spp. that produces propionate and *Butyrvibirio* spp. *Butyrvibirio fibrisolvens* are gram-negative, non-sporeforming, curved rods. These organisms mainly utilize mono- and disaccharides as substrates, resulting in the production of butyric acid (Bryant and Small, 1956).

Acetogenic Bacteria. The acetogenic bacteria primarily utilize propionate and butyrate as substrates within anaerobic digesters. *Syntrobacter wolinii* utilizes propionate and *Syntrophomonas wolfei* utilizes butyrate, where both organisms form acetate, CO_2, and H_2 (Wilkie and Colleran, 1987).

Methanogenic Bacteria. There are two primary groups of methanogenic bacteria active in anaerobic digesters: 1) hydrogen-utilizing methanogens and 2) acetate-utilizing methanogens. Hydrogenotrophic methanogens utilize CO_2, and H_2 for methane production. These organisms are not primary methane producers in the digesters, however their activity maintains a low partial pressure of hydrogen to drive interspecies hydrogen transfer within digesters. Acetotrophic methanogens (e.g., *Methanosarcina barkeri* and *Methanothrix soehngenii*) utilize acetate to form methane (CH_4). *Methanosarcina barkeri* is usually found in systems operating at low HRTs, whereas *Methanothrix soehngenii* is found in systems operating at high HRTs (Wilkie and Colleran, 1987). Acetate is the primary source of methane and accounts for

approximately 70% of the methane produced within digesters (Wilkie and Colleran, 1987).

Pathogen Decimation during Anaerobic Digestion of Animal Manure

Anaerobic digestion for stabilizing manure offers a non-chemical solution to control pathogenic organisms. Anaerobic digesters provide a controlled and optimum environment for the natural process of anaerobic degradation. The use of anaerobic technology to control pathogenic organisms has been studied for several systems. The survival of pathogenic organisms during anaerobic digestion of animal manure is influenced by several factors. Operating temperature and HRT have been shown to influence the rate of pathogen decimation during anaerobic digestion. However, environmental conditions during anaerobic digestion may also contribute to the rate of pathogen decimation. The pH of digester contents affects pathogen survival during digestion and systems have been designed to use this mechanism to reduce pathogen concentrations (Huyard et al., 2000). Substrate availability, microbial competition for substrates, and bacterial production of antibacterial compounds may also contribute to decimation of pathogenic organisms during anaerobic digestion (Kearney et al., 1993, Sahlstrom, 2003, Smith et al., 2005).

Temperature. One of the most important parameters influencing the rate of reduction of pathogenic organisms during anaerobic digestion is operating temperature. Temperature influences the kinetics of anaerobic digestion, which affects environmental conditions of digester contents. Anaerobic digesters are usually operated at either mesophilic (~35°C) or thermophilic (~55°C) temperatures.

Mesophilic operation maintains the digester at or near the temperature pathogens are exposed to in the animal. However, anaerobic digestion at this temperature range has

been shown to achieve significant reductions of pathogenic organisms. Duarte et al. (1992) demonstrated that mesophilic anaerobic digestion with a 15-day HRT resulted in a 3-$\log_{10}$ reduction of total coliforms, fecal coliforms, and streptococci from swine manure. Thermophilic operations maintain digester temperature in excess of that found within the animal. The higher operating temperatures have been shown to produce significant reductions of pathogens in shorter amounts of time as compared to mesophilic digestion (De Leon and Jenkins, 2002). Decimation times of pathogenic organisms are in the range of a few hours during thermophilic digestion, whereas mesophilic operations require several days (Berg and Berman, 1980, Burtscher et al., 1998, Watanabe et al., 1997). Chauret et al. (1999) found that mesophilic digestion of sewage sludge achieved a 0.3 $\log_{10}$ reduction of cyptosporida oocysts after 20 days. However, Kato et al. (2003) found thermophilic digestion of biosolids resulted in > 2 $\log_{10}$ reduction of cryptosporidia oocysts after 1 h.

Although thermophilic operation yields higher pathogen reductions, process stability has been cited as a disadvantage (Kim et al., 2002). Thermophilic operation results in rapid volatile acid production from fast-growing thermotolerant acidogens. The rapid production of volatile fatty acids lowers the pH of the medium, inhibiting the growth of methanogens, which grow at a slower rate. The low pH encountered in thermophilic operations reduces concentrations of pathogenic organisms.

Hydraulic Retention Time. The HRT of a digester greatly influences pathogen survival during anaerobic digestion. The HRT is the average amount of time a desired substrate remains in the system and is a function of system capacity and feed rate. The longer the HRT, the longer substrate is retained in the system, which lowers the amount

of substrate that can be treated per unit of time. Systems that operate at long HRTs (≥10 days) can achieve over 2 $\log_{10}$ reductions of pathogenic organisms from animal manure (Duarte et al., 1992). Pathogen survival is influenced by HRT, where retention in a suboptimum environment results in decimation. During the process of anaerobic digestion, environmental conditions become stressed due to lack of available nutrients and pathogens cannot maintain a high population density as compared to concentrations inside the animal.

Influence of Temperature and HRT. The combination of operating temperature and hydraulic retention time are regarded as the main factors impacting pathogen survival during anaerobic digestion. Mesophilic operations with long hydraulic retention times and thermophilic operations with short hydraulic retention times can reduce pathogen levels from animal manure. However, pathogen reduction during anaerobic digestion is not based solely on operating temperature and hydraulic retention time (Hill, 2003). Operating temperature and hydraulic retention time may influence the alteration of the physico-chemical properties of wastewater during digestion. Alteration of the physico-chemical properties may have direct impacts on pathogen acclimation and survival in an anaerobic digester environment.

Volatile Fatty Acids. Short-chain volatile fatty acids (e.g., acetate, propionate, and isobutyrate) produced by acidogens during anaerobic digestion can contribute to pathogen decimation. Abdul and Lloyd (1985) demonstrated growth inhibition of *E. coli* by various short-chain fatty acids. Cells were grown in 0.1% glucose under anaerobic conditions. Short-chain volatile fatty acids were added to the medium as potassium salts during early log phase. The authors demonstrated inhibition of growth by acetate (60 and

120 mM), propionate (52 and 104 mM), and isobutyrate (60, 90, and 180 mM). Kunte et al. (1998) demonstrated inactivation of *S. typhi* in cattle manure by volatile fatty acids during anaerobic digestion for 30 days (control) and 15 days (experimental). The feed for the experimental digester was supplemented with 1% glucose daily. The decimation times (T_{90}), the time required to achieve a 1 $\log_{10}$ reduction, were significantly lower for experimental digesters producing high volatile fatty acid concentrations (5 g/L) as compared to control digesters producing lower volatile fatty acid concentrations (0.1 g/L). However, the authors did not report operating temperature of each digester. Kwon and Ricke (1999) demonstrated growth inhibition of *Salmonella typhimurium* by propionic acid and sodium propionate. Cells were grown in tryptic soy broth (TSB) with various concentrations of propionic acid or sodium propionate. The culture medium was adjusted to pH 7.0. Inhibition of *S. typhimurium* was observed with propionic acid and sodium propionate concentration $\geq$ 25 mM.

Effect of pH. The pH of digester contents has significant effects on pathogen survival during anaerobic digestion. The production of short-chain volatile fatty acids during anaerobic digestion lowers the pH of the medium (Munch et al., 1998). Short-chain volatile fatty acids can contribute to pathogen decimation by lowering environmental pH. However, during anaerobic digestion, short-chain volatile fatty acids serve as substrates for subsequent methanogenesis. In a single-phase digester, where acidiogenesis and methanogenesis occur in the same reactor, the process of methanogenesis counteracts the pH effects of volatile fatty acids with the production of bicarbonate and the consumption of acetate. Bicarbonate buffers the digester contents, maintaining a pH of 6.5 to 7.0. Thus, pH may not contribute to pathogen decimation

during single-phase anaerobic digestion. Two-phase anaerobic digesters have been developed that separate the acidogenic phase from the methanogenic phase of anaerobic digestion. The acidogenic phase is usually operated at theromophilic (≥55°C) temperatures (Yu and Fang, 2000) and short HRTs (Huyard et al., 2000), resulting in rapid growth of thermotolerant acidogenic organisms. The effluent from the acidogenic phase is fed to the methanogenic phase, which is operated at mesophilic (~35°C) and long HRTs (≥ 10 days) to accommodate the slow growth rate of methanogens (Huyard et al., 2000). During the methanogenic phase, short-chain volatile fatty acids are converted to acetate, hydrogen, and carbon dioxide. These products serve as substrates for acetotrophic and hydrogenotrophic methanogens. Acidogenic and methanogenic phases can be operated and controlled independently, allowing for optimization of each phase without adverse affects to methanogenic and acidogenic populations (Ince, 1998). Phase separation allows for pathogen reduction during the acidogenic process due to lower pH resulting from the production of short-chain volatile fatty acids.

Solids Concentration. The survival of pathogens during anaerobic digestion of manure is influenced by the amount of solids present in digester contents. Pathogens can become readily associated with organic particles in the environment (Grossart et al., 2003). Nutrients absorb onto the surfaces of organic particles where pathogens can adhere and use the solids as a food source (Goulder, 1977). Bacterial adherence may be achieved by the use of fimbriae (Galfi et al., 1998), S-layers (Kotiranta et al., 1998), and pili (Orndorff et al., 2004). London-van der Waals forces, hydrophobic, and electrostatic interactions are the main mechanisms for bacterial and viral adhesion to solids (Ash, 1979, Daniels, 1980). In addition, exopolymeric substances and cell surface proteins also

contribute to adhesion to particles (Wicken, 1985). During anaerobic digestion, pathogens can attach to organic particles, supplying the organism with a nutrient source, increasing the likelihood of survival. Kumar et al. (1999) found decreased pathogen reductions from cattle dung using anaerobic digestion at mesophilic temperature with increased loading of volatile suspended solids concentrations.

Substrate Availability. The availability of substrate, as organic carbon, has a significant role in pathogen survival during anaerobic digestion. The amount of organic carbon available for biological degradation and assimilation is represented by biochemical oxygen demand (BOD). Anaerobic digesters can significantly reduce BOD concentrations in animal manure (Chin and Ong, 1993). The reduction in BOD creates a substrate-limiting environment, and pathogens are not readily suited to such stressed conditions. Pathogens associated with manure are voided from the intestines of the animal, an optimum environment. Once voided from the animal, pathogens in manure are exposed to environmental conditions. As manure enters an anaerobic digester, the amount of available substrate may fluctuate and pathogens have to be capable of adapting to changes in substrate fluctuations. The organic material that is available for biological degradation is mainly used for the formation of methane. During optimized anaerobic digestion, there is little available substrate to maintain pathogen concentrations found in the animal (Kearney et al., 1994).

Microbial Competition. Anaerobic digesters are optimum environments for methanogenesis and are operated for supporting the proliferation of organisms involved with the process of anaerobic digestion. Therefore, organisms involved with carbon flow through anaerobic digestion readily degrade and assimilate available organic carbon that

is fed to the system. Affinity for necessary substrates to support proliferation is higher for organisms involved in the process of anaerobic digestion than for pathogenic organisms. Saturation constants (K_s) for anaerobic digestion bacteria and substrates are lower than for other bacteria (Mosche et al., 1998). The lower K_s values result in a higher affinity for substrates for bacteria involved with anaerobic digestion (Song et al., 2004). Results from the aforementioned studies suggest that pathogens cannot readily utilize the limited amount of substrate to support a high population as compared with bacteria associated with anaerobic digestion, resulting in pathogen decimation.

In addition to higher affinity for substrates, differences in concentrations of digester microflora and pathogens influences microbial competition within anaerobic digesters. Pathogens are usually found at lower concentrations than nonpathogenic bacteria, unless voided in high numbers by an infected animal (Huston et al., 2002). Anaerobic digesters are predominated by bacteria involved with anaerobic digestion and are present in high concentrations (10^7 to 10^8 CFU/ml) (Solera et al., 2001). Pathogens are usually loaded to the system in lower concentrations as compared to anaerobic digestion bacteria already present in the system. The difference in population density and the higher affinity for substrates give anaerobic digestion bacteria a survival advantage in anaerobic digesters, leaving little opportunity for bacterial indicators and pathogens to become established.

Antimicrobial Products of Bacteria. Bacteria are in constant competition with other bacterial species for available nutrients in natural environments. Different genera of bacteria produce substances that have antibacterial properties. The type of substances produced and the resulting effects can be specific for different species within the same genus. Bacteriocins and colicins are antimicrobial compounds that are produced by

bacteria to give the organism a competitive advantage in natural environments. The role of bacteriocins and colicins has not been extensively studied in regards to pathogen reduction during anaerobic digestion. However, organisms that can be found in animal manure and subsequently in anaerobic digesters have been shown to produce bacteriocins and colicins. Holo et al. (2002) reviewed bacteriocins from propionate producing bacteria that were effective inhibitors of gram positive and gram negative organisms. Lyon and Olson (1997) isolated colicins produced by *Escherichia coli* ECL12 (isolated from bovine feces) that inhibited the growth of pathogenic *E. coli* strains. Bacteriocins and colicins may be present and active in digesters, contributing to pathogen decimation during anaerobic digestion.

Summary

Pathogenic organisms present in animal manure pose a serious herd and human health concern. Manure management practices have become the focus of recent legislation to control the transmission of zoonotic organisms to the environment. Anaerobic digestion has been shown to reduce the level of pathogens from animal manure. The rate of pathogen decimation during anaerobic digestion has been related to operating temperatures and hydraulic retention times. However, other factors, such as pH, volatile fatty acid concentrations, microbial competition, and substrate affinity, have a significant role in the fate of pathogenic organisms during anaerobic digestion. Although systems designed for the purpose of pathogen reduction focus on operating temperature, reactor configurations that can optimize other factors that contribute to pathogen reduction may prove efficient and superior to current systems for manure wastewater sanitization.

Purpose of Study

The purpose of the current work was to identify the factor(s) that contribute to the reduction of indicator and pathogenic bacteria during anaerobic digestion in a fixed-film system. Also, we sought to study the transport of residual viruses through soil following land application of anaerobically treated flushed dairy manure wastewater.

CHAPTER 3
INDICATOR AND PATHOGENIC BACTERIA REDUCTION BY ANAEROBIC DIGESTION: THE ROLE OF MICROBIAL COMPETITION AND SUBSTRATE LIMITATIONS

Introduction

Several pathogenic bacteria are known to be associated with dairy manure. These include organisms that mainly infect dairy cattle, such as *S. aureus* and streptococci, and zoonotic organims, such as *E. coli* O157:H7 and *Salmonella* spp., which can cause infections in humans. Therefore, disposal of animal manure by land application raises environmental and public health concerns. Effective treatment of manure by anaerobic digestion can be achieved using well-managed systems. Anaerobic digesters have been shown to achieve 2 to 3 $\log_{10}$ reductions of a variety of indicator and pathogenic organisms from various substrates (DeLeon and Jenkins, 2002, Duarte et al., 1992). Decimation of pathogenic bacteria during anaerobic digestion may due to inhibitory effects caused by microbial interactions and competition for limited substrates.

Inhibitory effects of indigenous microflora may involve the production of secondary metabolites. Such metabolites are produced during stationary phase or idiophase and usually have antimicrobial properties (Riviere et al., 1975). Secondary metabolites include antibiotics, colicins and bacteriocins. These compounds are usually effective against strains related to the producing organism. Lyon and Olson (1997) studied a bacteriocin, designated colicin ECL 12, from *E. coli* isolated from swine manure. Colicin ECL 12 was found to inhibit two strains of *E. coli* and 17 strains of *E. coli* O157:H7. However, there were no inhibitory effects found against selected gram-

positive pathogenic bacteria (e.g. *S. aureus* and *L. monocytogenes*). Bacteriocins may also demonstrate activity against both gram-positive and gram-negative organisms. Hyronimus et al. (1998) found that a strain of *Bacillus coagulans,* isolated from bovine manure, produced coagulin. Coagulin is a bacteriocin capable of inhibiting a variety of bacteria including strains of *Bacillus* spp., *Enterococcus* spp. and *Listeria* spp. Propionic bacteria have also been shown to produce bacteriocins. Holo et al. (2002) identified several bacteriocins produced from propionate producing bacteria.

Volatile fatty acids (VFAs) produced by bacteria have also been shown to display bacteriostatic or bacteriocidal effects. Kunte et al. (1998) measured the effects of VFA concentrations on the decimal reduction time (T_{90}) of *Salmonella typhi* in anaerobic digesters. The authors used a KVIC model floating dome digester (9.5 L) operated at a 15-day retention time and fed cattle dung slurry (6% total solids). The investigators found that at a constant total VFA concentration (5000 mg/L) there was a rapid reduction in *S. typhi* initially, but the numbers of the organism eventually leveled off and maintained a concentration of 10^2 cells per ml. The authors reported a decrease in the decimal reduction times between the control (T_{90}, 4.22 days; VFA concentration, 100 - 125 mg/L) and experimental (T_{90}, 18.63 days; VFA concentration, 4800 - 5700 mg/L) digesters. The study, however, did not determine the concentration of specific VFAs. Kwon and Ricke (1999) specifically studied the effect of propionate on *S. typhimurium* as a pure culture under anaerobic conditions. Details of the study are given previously (*see* Chapter 2). Complete growth inhibition of *S. typhimurium* was observed at a propionate concentration of 1830 mg/L. Sensitivity to VFAs under anaerobic conditions has also been demonstrated with *E. coli*. Abdul and Lloyd (1985) showed that, under anaerobic

conditions, *E. coli* was inhibited by increasing concentrations (60 to 180 mM) of acetate, propionate, and isobutyrate. The experimental conditions are described previously (*see* Chapter 2).

Volatile fatty acid concentrations may contribute to indicator and pathogenic organism decimation during anaerobic digestion. However, during anaerobic digestion, VFAs, particularly acetate, serves as substrates for methanogenesis. Sooknah and Wilkie (2004) found that the concentrations of acetate in the effluent of a fixed-film digester, operated at a 2-day HRT and ambient temperature, treating flushed dairy manure wastewater was below 170 mg/L or undetectable. Therefore, VFAs may not contribute to indicator and pathogenic bacteria reduction during anaerobic digestion in a fixed-film system.

The contribution of the indigenous microflora in the liquid phase during fixed-film anaerobic digestion to indicator and pathogenic bacteria reduction may include the production of antimicrobial compounds, more so than the production of VFAs. However, competition between the indigenous microflora and indicator and pathogenic bacteria may also occur. Thus, the role of indigenous microflora in the liquid phase during fixed-film anaerobic digestion remains unclear.

Purpose

The purpose of the current experiment was to determine if the reduction of indictor and pathogenic bacteria in the liquid phase of fixed-film anaerobic digestion at ambient temperature is attributed to the presence of indigenous microflora, inhibition of growth by digester contents, or by starvation.

Materials and Methods

Dairy Research Unit

The University of Florida Dairy Research Unit (DRU) is located in Hague, FL. The facility has an average milking herd of 500 cows, which are housed and fed in free stall barns. These barns are constructed with slanted concrete floors to allow downward flow of the flush water to remove manure and urine. Sand is used as bedding in the free stall barns instead of organic bedding materials because sand is inorganic and limits bacterial growth.

Manure Handling

The manure handling process starts with flushing of the free stall barns and the milking parlor. Manure is removed by intermittent, automated flushing. Sand is removed by sedimentation in the sand trap and fibrous solids are removed using a mechanical solids separator. The remaining solids are further removed by sedimentation basins. The resulting slurry (influent) flows through a bar-screen before entering the pump sump.

Fixed-film Anaerobic Digester

The fixed-film anaerobic digester used in the study is a demonstration-scale unit with a 100,000 gal total capacity (97,377 gal active liquid volume). The system was operated at ambient temperature and an average HRT of 3-days. The system is located past the free stall barns and adjacent to the waste storage pond. The digester is equipped with a flare and is used to convert excess methane to CO_2 and H_2O, to avoid methane emissions. Influent is fed into the digester from the pump sump. The material is recycled through inert media to be degraded by the attached biofilm. Anaerobically digested effluent is stored in waste storage ponds before being applied to land.

Sample Collection, Characterization, and Preparation

Sample Collection. Influent and effluent was collected from the DRU. Influent was collected as three 1 L samples from the pump sump. Effluent was collected as three 1 L samples from a sampling port located on the side of the fixed-film reactor. The port is used for taking samples of the effluent prior to exiting the system. Sample temperature was measured at the time of collection.

Characterization. The samples were characterized by measuring the following parameters according to standard methods (APHA, 1998): pH (section 4500-H B), total solids (TS, section 2540 B), volatile solids (VS, section 2540 E), suspended solids (SS, section 2540 D) and volatile suspended solids (VSS, section 2540 E). Total chemical oxygen demand (TCOD) was performed on the whole fraction of samples using COD tubes (Hach Co. Loveland, CO). Soluble chemical oxygen demand (SCOD) was performed on the soluble fraction of wastewater. The soluble fraction was obtained by centrifugation at 11,000 x g for 30 minutes and filtration through a 0.45 μm nitrocellulose filter (Millipore). A 5-day biochemical oxygen demand (BOD_5) was measured on the whole ($TBOD_5$) and soluble ($SBOD_5$) fractions of the samples using a BODTrak® apparatus (Hach Co., Loveland, CO). To prevent nitrification, 175 mg 2-chloro-6 trichloromethyl pyridine (N-Serve # 253335, Hach Co., Loveland, CO) was added to each BOD bottle.

Microbial Analysis. The microbial population (i.e., anaerobes, aerobes, and facultative organisms) was determined. Total anaerobes and aerobes were measured by direct plating onto plate count agar (Difco). Plating and incubation for total anaerobes was performed in the anaerobic chamber (described later). All plates were incubated at

room temperature for 5 days. Total facultative organisms were determined by most probable number (MPN) technique using fluid thioglycollate (Difco) tightly capped and incubated as previously stated using aerobic conditions.

Organic acids and sugars were measured in the soluble fractions of influent and effluent. The samples (100 ml) were ionized by adjusting to pH 11 with 5 N NaOH. The samples were then concentrated using a microrotary film evaporator (Evapotec$_{TM}$, #421-4000, Labconoco, Lenexa, KS) at 100°C. Concentrated samples were reconstituted with deionized water, using 3 ml for influent and 2 ml for effluent. Reconstituted samples were then filtered through a 0.45μm nitrocellulose filter (Millipore). The filtered samples were analyzed using a Hewlett-Packard HPLC (HP 1090 Series II) equipped with a refractive index (RI) detector and a UV monitor (210 nm).

Bacterial Cultures

Escherichia coli, *Enterococcus* spp., *S. aureus*, and *Salmonella* spp. used in the study were isolated previously from influent. *Escherichia coli* O157:H7 (ATCC 43888) was obtained from the American Type Culture Collection. All cultures were grown in 3% (w/v) tryptic soy broth (TSB) overnight at 37°C. The cells were harvested by centrifugation at 2,000 x g for 10 minutes and washed three times with sterile 1X phosphate buffered saline, pH 7.5 (PBS). The cells were resuspended in 10 ml PBS and stored at 5°C until use.

Bacterial Quantification

Bacterial concentrations were determined by direct plate counts using mFC agar, mE agar, mannitol salt agar (acriflavine added for whole fractions), and XLD agar for *E. coli* (and fecal coliforms), *Enterococcus* spp., *S. aureus*, and *Salmonella* spp., respectively. All media was prepared according to the manufacturer's instructions.

Enterococcus spp. and *S. aureus* were incubated at 37°C for 48 hours. Fecal coliforms and *E. coli* were incubated at 45°C overnight. *Salmonella* spp. was incubated at 37°C for 24 hours. Total anaerobes and aerobes were quantified as described previously.

Anaerobic Conditions

Anaerobic conditions during incubation were maintained in an anaerobic chamber (Coy, Grass Lake, MI), which was filled with a gas mixture of 80% N_2 and 20% CO_2. Residual oxygen was removed from the chamber using hydrogen and palladium catalysts. Pipettes and equipment were stored within the chamber to minimize oxygen contamination. The chamber was also equipped with a forced air incubator.

Survival in Whole and Soluble Wastewater Fractions

The soluble fraction of influent was used to expose the test organisms to the substrates present in the liquid phase during feeding of the digester. The soluble fraction of effluent was used to expose the test organisms to the substrates that would be present in the liquid phase during digestion. The soluble fractions of influent and effluent were obtained as previously described in "Sample Collection, Characterization, and Preparation." The soluble fractions were transferred as 5 ml aliquots in triplicate to sterile, capped 13mm test tubes. The samples were inoculated individually with each test organism, prepared as previously described. The samples were then transferred to the anaerobic chamber and incubated for 3 days at 28°C. Positive and negative controls were 3% TSB and PBS, respectively. Total anaerobes, aerobes, facultative organisms, and test organisms were measured as previously described.

The whole fraction of influent was used to expose the test organisms to the indigenous population that would be present in the liquid phase during feeding of the digester. The whole fraction of effluent was used to expose the test organisms to the

indigenous population that would be present during digestion of the liquid phase. The whole fractions of influent and effluent were transferred as 5 ml aliquots in triplicate into sterile, capped 13mm test tubes and screw-cap tubes containing a magnetic stirring bar. The tubes were transferred to the anaerobic chamber and incubated for 3 days at 28°C (without mixing) and 38°C (mixed). Total anaerobes, aerobes, facultative organisms, fecal coliforms, *Enterococcus* spp., and *S. aureus* were measured as previously described. The survival of *E. coli* O157:H7 and indigenous *Salmonella* spp. was not studied in experiments with the whole fraction. Due to the low occurrence of these pathogens in the wastestream of the DRU, artificial inoculation would have been required and potentially would hinder simulating the natural population encountered by indigenous fecal coliforms, *Enterococcus* spp. and *S. aureus*.

Inhibition and Wastewater Supplementation Studies

Inhibition effects were determined by supplementing the soluble fractions of influent and effluent with 0.09% TSB. The amount of TSB used was the minimal concentration that could support the growth of *S. aureus*. Soluble influent and effluent, and supplemented soluble fractions were inoculated with a suspension of *S. aureus* prepared as previously described. Negative and positive controls were PBS and 0.09% TSB, respectively. The samples were transferred to the anaerobic chamber and incubated under for 3 days at 28°C. *Staphylococcus aureus* were quantified as previously described.

Effluent was supplemented with a carbon source and yeast extract (YE) to determine the effect of substrate limitation on the proliferation of *S. aureus*. Effluent was supplemented with 5 and 55 mM glucose, 0.1% YE, and ashed YE. Glucose served as a sole source of carbon and YE served to represent organic carbon and nutrients that may

be present in manure from cellular debris. The use of 55 mM glucose was based on the carbohydrate concentration of commercial growth media (e.g., mannital salt agar, Difco). The percentages of carbon in 5 mM and 55 mM glucose was equivalent to the percentage of carbon in 0.1% YE (Table 3-1). Ashed YE served as a source of inorganic nutrients and minerals. Ashed YE was obtained by ignition of 5g YE at 550°C for 4 hrs. The residue following ignition was added to the effluent at the same amount present in 0.1% YE. Soluble wastewater, supplemented soluble wastewater, and controls (i.e., PBS, 3% TSB, and influent) were inoculated with *S. aureus* suspended in PBS. The samples were transferred to the anaerobic chamber, incubated, and assayed for *S. aureus* as previously described.

Statistical Analysis

Log_{10} increases or decreases were determined under each condition by subtracting the final log_{10} transformed concentration from the initial log_{10} transformed concentration. Each condition was compared with the positive control and negative control using one-way ANOVA and Duncan's Method with a significance level of 0.05. The statistical analysis was performed using ProStat© v 3.5.

Results

Wastewater Characteristics

The properties of the wastewater samples used in the study are given in Table 3-2. Anaerobic treatment reduced TCOD by 52%, SCOD by 72%, $TBOD_5$ by 62%, and $SBOD_5$ by 57%. Total aerobes, anaerobes, and facultative organisms were reduced by 62%, 73%, and 19%, respectively. Acetate and acetoin were reduced by greater than 80%.

Growth of Bacteria in Whole and Soluble Fractions of Wastewater

To determine if the indigenous microflora affected the concentrations of indicator bacteria (fecal coliforms and *Enterococcus* spp.) and pathogenic bacteria (*S. aureus, Salmonella* spp. and *E. coli* O157:H7), influent and effluent with and without indigenous microflora (i.e., whole and soluble fractions, respectively) were incubated under anaerobic conditions for 3 days at 28°C.

A. Total aerobic, anaerobic, and facultative bacteria

Soluble wastewater fraction. Growth of total aerobic, anaerobic, and facultative bacteria in the soluble fractions of influent and effluent is shown in Table 3-3. Log_{10} increases in concentration of all groups were significantly lower ($P < 0.05$) in influent than in 3% TSB. However there was no significant difference ($P < 0.05$) between the log_{10} increases in concentration between 3% TSB and effluent for all three groups. Furthermore, there was more growth of all groups in soluble effluent than in soluble influent. Although growth of all groups was lower in the influent, the log_{10} increase for both groups was significantly higher ($P < 0.05$) than the other organisms used in the study. This trend is also observed with effluent.

Whole wastewater fraction. Log_{10} decreases in concentrations of indigenous total anaerobic, aerobic, and facultative bacteria were observed in the whole fractions of influent and effluent at 38°C and 28°C (Table 3-4). Unlike tests with the soluble fractions where 10 to 20 µl of wastewater was used as an inoculum, the whole fractions were incubated without any alterations. Indigenous concentrations of all groups may have already been at a maximum in the whole fractions, where further incubation resulted in a decline in population. The log_{10} reduction of total anaerobes between 38°C and 28°C in influent was not significantly different ($P < 0.05$). This trend was also observed in the

effluent. The $\log_{10}$ reduction for total anaerobic bacteria was significantly lower ($P < 0.05$) in effluent at 38°C and 28°C than in influent at the same temperatures. Conversely, the $\log_{10}$ decrease for total aerobic bacteria was significantly lower ($P < 0.05$) in influent at 38°C and 28°C and in effluent at 38°C than in effluent at 28°C. Also, the reduction of total aerobes in influent at 38°C and 28°C was significantly lower ($P < 0.05$) than for total anaerobes in influent at the same temperature. The reduction of total facultative organisms at 38°C in influent and effluent and 28°C in influent was significantly higher than at 28°C in effluent. Furthermore, the reduction of total anaerobes and facultative organisms in effluent at 28°C was significantly lower ($P < 0.05$) than for total aerobes, fecal coliforms, *Enterococcus* spp, and *S. aureus* (Table 3-5). These results suggest that the anaerobic and facultative population is more stable and active in the effluent.

B. *E. coli* and fecal coliforms

Soluble wastewater fraction. *Escherichia coli* isolated from influent was chosen as a representative of the fecal coliform population in influent and effluent. This organism was able to proliferate in the soluble fractions of influent and effluent (Table 3-3). However, growth in both fractions was significantly lower ($P < 0.05$) than in the positive control. Furthermore, growth of this strain of *E. coli* in effluent was significantly lower ($P < 0.05$) than in influent. This result shows that effluent is limited in the amount of available substrate to support significant increases in concentration.

Whole wastewater fraction. In the whole fractions of influent and effluent, fecal coliforms were not detected after incubation at 38°C or 28°C (Table 3-4). This result shows that in presence of indigenous total aerobic and anaerobic organisms, fecal coliforms were not able to survive in influent or effluent at either temperature.

C. *Enterococcus* spp.

Soluble wastewater fraction. *Enterococcus* spp. isolated from influent was able to proliferate in both soluble fractions of influent and effluent (Table 3-3). There was no significant difference ($P < 0.05$) between the $\log_{10}$ increase in influent and the positive control. However, the $\log_{10}$ increase in effluent was significantly lower ($P < 0.05$) than influent and the positive control. These results show that anaerobic treatment reduces the amount of available substrate to support significant increases in concentration of indigenous *Enterococcus* spp.

Whole wastewater fraction. There was significantly more reduction of *Enterococcus* spp. in influent at 38°C than at 28°C (Table 3-4). Also, there was significantly more reduction in influent at both temperatures than in effluent. The reduction of *Enterococcus* spp. in effluent at 38°C and 28°C was not significantly different ($P < 0.05$) (Table 3-4). There were significantly more reduction of *Enterococcus* spp. in influent at both temperatures than for total aerobes and anaerobes (Table 3-5). In the effluent at both temperatures, the reduction of *Enterococcus* spp. was significantly higher ($P < 0.05$) than for total anaerobes (Table 3-5). In the whole fractions of influent and effluent, the $\log_{10}$ reductions of *Enterococcus* spp. were 1.04 and 0.69, respectively (Table 3-4). This result shows that the indigenous microflora in influent and effluent contributes to the reduction of this organism during the digestion process. These results also suggest that mesophilic temperatures are not required to reduce *Enterococcus* spp.

D. *Salmonella* spp.

Soluble wastewater fraction. *Salmonella* spp. isolated from influent was able to proliferate in the soluble fractions of influent and effluent (Table 3-3). However, growth in both fractions was significantly lower ($P < 0.05$) than the positive control.

E. *E. coli* O157:H7

Soluble wastewater fraction. *Escherichia coli* O157:H7 was able to proliferate in the soluble fractions of influent and effluent (Table 3-3). Also, there was no significant difference ($P < 0.05$) between the $\log_{10}$ increases in concentrations for the positive control, influent and effluent.

Differences in growth were observed between pathogenic and nonpathogenic *E.coli* in the soluble fractions of influent and effluent. Growth in influent was similar to that of indigenous *E. coli.* However, the $\log_{10}$ increase in effluent for *E. coli* isolated from influent was significantly lower ($P < 0.05$) than for *E. coli* O157:H7.

F. *S. aureus*

Soluble wastewater fraction. There was no significant difference ($P < 0.05$) in the growth of indigenous *S. aureus* in the soluble fraction of influent and the positive control (Table 3-3). There was also no significant difference ($P < 0.05$) between the $\log_{10}$ decreases in effluent and the negative control. This shows that *S. aureus* can proliferate in influent but not effluent.

Whole wastewater fraction. Reductions of *S. aureus* in influent and effluent at 38°C and 28°C were similar (Table 3-4). Also, the reduction of *S. aureus* in effluent at 38°C and 28°C was not significantly different ($P < 0.05$). These results suggest that digestion at both temperatures result in similar decreases of *S. aureus* in effluent.

However, there was significantly more reduction of *S. aureus* in influent at 38°C than at 28°C.

The reduction of *S. aureus* in effluent at 38°C and 28°C was significantly higher ($P < 0.05$) than for total anaerobes and aerobes (Table 3-5). However, the reductions of *S. aureus* observed in the whole fraction of effluent at both temperatures (-0.84) were less than those observed in the soluble fraction (-1.43) at 28°C. These results suggest that either inhibitory compounds or nutritional limitations adversely impact *S. aureus*.

Inhibition or starvation of *S. aureus*. To determine if inhibitory compounds or substrate limitations impacted *S. aureus*, soluble effluent was supplemented with a minimal concentration of TSB. As previously observed, *S. aureus* was not able to proliferate in effluent (Table 3-6). However, supplementation with TSB resulted in a 1.6 $\log_{10}$ increase in concentration. This result suggests that proliferaton of *S. aureus* is restricted by substrate limitations, not inhibitory compounds.

Wastewater supplementation. Supplementation resulted in proliferation of *S. aureus* in soluble effluent (Table 3-7). Log_{10} increases of *S. aureus* in effluent supplemented with 55 mM glucose solution were significantly higher ($P < 0.05$) than in the other solutions tested. Growth was not observed in effluent or 5mM and 55 mM glucose solutions. However, addition of 5 mM and 55 mM glucose to effluent resulted in proliferation of *S. aureus*. Also, there was no growth detected in effluent supplemented with ashed yeast extract, suggesting that inorganic nutrition is not the limiting substrate. These results demonstrate that the inability of *S. aureus* to proliferate in the effluent is due to the lack of sufficient sources of carbon.

Discussion and Conclusions

Anaerobic digestion is known to reduce the concentrations of pathogenic bacteria from various manures. The decimation is commonly attributed to operating temperature and HRT, where higher temperatures and longer HRTs result in higher rates of decimation. However, manure contains a diverse microbial population and the influence of the indigenous microflora in manure on the survival of indicator organisms and pathogenic bacteria has not been studied extensively. Few studies have investigated the influence of indigenous microflora on pathogenic bacteria in manure, under anaerobic conditions. Shin et al. (2002) found that a fecal suspension (FS) and its anaerobic culture (FC) displayed inhibitory effects on *E. coli* O157:H7. An overnight culture of *E. coli* O157:H7 (1×10^5 CFU/ml) was grown in mixed culture with FS (5×10^8 CFU/ml) and FC (5×10^8 CFU/ml) anaerobically for 24 h at 37°C. Growth of these pathogenic bacteria was inhibited in the presence of FS and FC. Also, decimation of *E. coli* and *Salmonella* spp. during mesophilic anaerobic digestion of sewage sludge (20 day HRT) has been attributed to competition with indigenous microflora (Smith et al., 2005).

Similar impacts of indigeous microflora have been demonstrated against *S. aureus*. Donnelly et al. (1968) studied the proliferation and enterotoxin production of *S. aureus* (10^4 CFU/ml) under aerobic conditions in raw milk with a high ($> 10^6$ CFU/ml) and low ($< 10^4$ CFU/ml) standard plate count (SPC, i.e., indigenous microflora). At 30°C, *S. aureus* was able to proliferate and produce toxin in low SPC milk. However, in high SPC milk at the same temperature, *S. aureus* demonstrated less proliferation and no enterotoxin was detected. The authors concluded that *S. aureus* was adversely impacted by the activity of indigenous microflora. In the current study, the indigenous microflora in wastewater (10^7 CFU/ml) caused decimation of native *S. aureus* (10^3 CFU/ml).

DiGiacinto and Frazier (1966) demonstrated inhibited growth of *S. aureus* during incubation with coliforms and *Proteus* spp. under aerobic conditions in TSB. *Eschericihia coli* (2 x 10^4 CFU/ml) and *Proteus vulgaris* (2 x 10^4 CFU/ml), both commonly found in animal manure, were found to inhibit the growth of *S. aureus* (2 x 10^4 CFU/ml) in mixed cultures at 22°C and 30°C. Results from the current study showed significant reduction of fecal coliforms (> 2 $\log_{10}$) in the presence of indigenous microflora, suggesting their competition with *S. aureus* may be minimal. However, competition between *S. aureus* and *Proteus* spp. during fixed-film anaerobic digestion is not known.

The current study suggests that the indigenous microflora responsible for decimation of indicator and pathogenic bacteria may consist of strict anaerobic and facultative organisms. Total anaerobic and facultative organisms were reduced significantly less than fecal coliforms, *Enterococcus* spp., and *S. aureus*. These results suggest that the activity of the anaerobic and facultative population in the liquid phase contributes to the reduction of indicator and pathogenic bacteria during fixed-film anaerobic digestion.

In the current study, the test organisms, with the exception of *S. aureus*, were able to proliferate in the soluble fraction of effluent, whereas growth did not occur in the whole fraction of effluent. The difference in growth may be attributed to the ability of the test organisms, as a pure culture, to use the remaining carbon sources (e.g., acetate and acetoin). The concentration of the remaining acetate (1.1 mM) and acetoin (0.4 mM) may have been too low to support the proliferation *S. aureus*.

Exposure to optimum growth temperature of bacterial pathogens, in the presence of indigenous microflora, does not increase the proliferation of pathogenic bacteria (Donnelly et al., 1968). Similarily, in the current study, the reductions of indicator and pathogenic bacteria observed in the whole fraction of effluent at 38°C and 28°C were similar. These results suggest that anaerobic digestion at optimum growth temperatures of indicator and pathogenic bacteria will not increase survival in the presence of indigenous microflora.

Limited substrate concentration was found to specifically affect *S. aureus*. In the current study, *S. aureus* was the only test organism that could not proliferate in the soluble fraction of effluent, where growth was observed in the soluble fraction of influent. Furthermore, a higher reduction was observed in the soluble fraction of effluent than in the whole fraction of effluent, suggesting that removal of nutrients or the presence of inhibitory compounds, resulting from the activity of indigenous microflora, influenced reduction of *S. aureus* during incubation. Supplementation with tryptic soy broth demonstrated that starvation, and not inhibitory compounds, were preventing proliferation of *S. aureus*. This finding suggests that substrates necessary to support *S. aureus* were removed during anaerobic treatment of influent, resulting in starvation of *S. aureus*. Furthermore, because *S. aureus* was the only test organism that did not grow in the soluble fraction of effluent, the use of the tested indicators to predict the survivability of *S. aureus* following digestion may not be valid. Therefore, direct monitoring of *S. aureus*, as apposed to using surrogates, may be necessary.

Starvation of *S. aureus* during anaerobic digestion may be induced by the inability to compete with the indigenous microflora. The inability to compete with the indigenous

microflora may be attributed to the differences in concentrations in native wastewater. The indigenous population was present at 10^6 to 10^7 CFU/ml and *S. aureus* was present at 10^2 to 10^3 CFU/ml. Furthermore, during digestion, there was a low reduction (19%) of facultative organisms, suggesting their stability in the liquid phase during digestion. Thus, *S. aureus* may have been out competed for available substrates, resulting in starvation.

Although *S. aureus* may be reduced by starvation, survival of the residual population during digestion may be possible. In stressed conditions, *S. aureus* enters a starvation-survival state. The starvation state of *S. aureus* was characterized by Clements and Forster (1998). Differential protein expression and morphological changes of *S. aureus* were observed after the cells were exposed to long-term glucose limiting conditions. The morphological changes associated with the starvation state included reduction in cell size and lack of division septa. Several different proteins not normally observed in vegetative cells were expressed prior to and during the starvation state. This finding suggested that *S. aureus* utilized differential protein expression to enter the starvation state and that the cells were not dormant during the starvation state. Also, during starvation conditions, a 3 $\log_{10}$ reduction of vegetative *S. aureus* was observed before entry into the starvation state. The investigators found that the recovery of *S. aureus* from a starvation state could not occur by the addition of solely glucose or amino acids. A combination of glucose and amino acids were required to induce growth. Also, an unknown factor (or factors) present in media utilized by vegetative *S. aureus* cells was found to induce growth of starved cells.

Another phenotypic trait of starvation-state *S. aureus* is the formation of smaller colonies, as compared to normal vegetative cells, that are similar to small colony variants (Clements et al., 1999). Small colony variants of *S. aureus* are mutations that arise from stressed conditions. The mutations result in decreased coagulase and hemolysin activity, slow growth, reduced or no pigmentation, and small colony size (diameter < 0.1 mm) on agar media (Clements and Foster, 1999, Kaplan and Dye, 1976).

In the current study, the results demonstrated proliferation of *S. aureus* in the soluble fraction of effluent following the addition of only carbon. This result shows that *S. aureus* was starved for carbon and that carbon is a growth-limiting constituent in effluent. During anaerobic digestion, carbon is converted mainly to CH_4, CO_2, and biomass. The reduced COD of the effluent demonstrated the reduction of carbon during anaerobic digestion. The reduced COD suggests that utilization of carbon by other indigenous microflora during anaerobic digestion resulted in starvation of *S. aureus*. Entry into a starvation stated during anaerobic digestion may occur when the organism becomes stressed.

The current study shows the reduction of indicator and pathogenic bacteria in the liquid fraction is due to microbial competition and starvation from the lack of sufficient carbon sources. These findings suggest possible mechanisms for the decimation of these organisms during anaerobic treatment. The current study showed reductions of these organisms in the presence of indigenous microflora at ambient temperature (28°C) and a low HRT (3 days). The results of this study demonstrate the importance of indigenous microflora in manure for sanitization of manure wastewater by anaerobic digestion. Furthermore, removal of carbon sources necessary to support pathogenic bacteria may

play a role in decimation. Also, the use of indicators to predict the presence of *S. aureus* should be reevaluated.

Table 3-1. Percentage of carbon from various sources in yeast extract.

Component*	# Carbons	Mol. Wt.	% Carbon	% component in YE	% Carbon from component in YE
Ala	3	89	40.48	5.36	2.17
Arg	6	174	41.41	3.02	1.25
Cys	3	121	29.78	0.74	0.22
Asp	4	133	36.12	6.69	2.42
Glu	5	147	40.85	14.20	5.80
Gly	2	75	32.03	3.25	1.04
His	6	155	46.49	1.20	0.56
Ile	6	131	55.01	3.23	1.78
Leu	6	131	55.01	4.69	2.58
Lys	6	146	49.36	5.15	2.54
Met	5	149	40.30	1.05	0.42
Phe	9	165	65.51	2.53	1.66
Pro	5	115	52.22	2.60	1.36
Ser	3	105	34.31	2.84	0.97
Thr	4	119	40.37	2.95	1.19
Trp	11	204	64.76	1.36	0.88
Tyr	9	181	59.72	1.20	0.72
Val	5	117	51.32	3.79	1.95
carbohydrates	6	180	40.03	17.50	7.01
Biotin	10	244.31	46.19	0.0003	0.00015
Choline chloride	5	139.63	43.01	0.03	0.013
folic acid	19	441.4	51.70	0.0002	0.00008
Inositol	6	180.16	40.00	0.14	0.056
nicotinic acid	6	123.11	58.53	0.060	0.035
PABA	7	137.00	47.98	0.076	0.037
Pantothenic acid	9	219.23	49.30	0.027	0.014
Pyridoxine	8	205.64	46.72	0.004	0.002
Riboflavine	17	376.36	54.25	0.012	0.006
Thiamine	12	300.81	42.73	0.053	0.023
thymidine	10	242.23	49.58	0.002	0.0009
TOTAL % C in YE					**36.69**
Amount (%C) in 1% YE					0.367
Amount (%C) in 55 mM carbon source					0.390
Amount (%C) in 0.1% YE					0.037
Amount (%C) in 5 mM carbon source					0.036

*The components of yeast extract (YE) were obtained from a typical analysis of YE (Difco Laboratories, 1998).

Table 3-2. Characterization of samples used for all experiments.

Parameter*	Influent	Effluent
Temperature, °C	24	25
pH	7.55	7.06
TCOD, mg/L	3707 ± 15**	1779 ± 31
SCOD, mg/L	2083 ± 38	590 ± 4
TS, mg/L	3904 ± 169	3064 ± 55
SS, mg/L	1136 ± 69	1034 ± 69
VS, mg/L	2352 ± 114	1543 ± 40
VSS, mg/L	920 ± 22	780 ± 65
$TBOD_5$, mg/L	3413 ± 175	1313 ± 64
$SBOD_5$, mg/L	1046 ± 5	448 ± 33
Total aerobes, CFU/ml	$2.1 \pm 0.2 \times 10^7$	$7.9 \pm 1.0 \times 10^6$
Total anaerobes, CFU/ml	$1.3 \pm 0.4 \times 10^7$	$3.4 \pm 1.1 \times 10^6$
Total facultative, MPN/ml	$9.4 \pm 0.3 \times 10^6$	$7.6 \pm 0.5 \times 10^6$
Acetate, mM	6.3 ± 0.7	1.1 ± 0.07
Acetoin, mM	2.1 ± 0.6	0.42 ± 0.02

Notes: Anaerobically digested flushed dairy manure wasetewater (effluent) was obtained from the University of Florida IFAS fixed-film anaerobic digester. Flushed dairy manure wastewater (influent) was material collected prior to anaerobic treatment.

*Parameter abbreviations are as follows: TCOD = total chemical oxygen demand, SCOD = soluble chemical oxygen demand, TS = total solids, SS = suspended solids, VS = volatile solids, VSS = volatile suspended solids, $TBOD_5$ = 5-day total biochemical oxygen demand and $SBOD_5$ = 5-day soluble biochemical oxygen demand.

**Values are average ± standard deviation (n = 3).

Table 3-3. Growth of indicator and pathogenic bacteria in the soluble fraction of wastewater.

Organism	Initial conc, CFU/ml	Log_{10} Reduction			
		3% TSB	1X PBS	Influent	Effluent
Total aerobes	$3.6 \pm 0.6 \times 10^4$	4.10^A	NA	2.60^B	3.99^A
Total anaerobes	$1.7 \pm 0.2 \times 10^4$	3.54^A	NA	2.86^B	3.63^A
Total facultative, MPN/ml	$8.3 \pm 0.3 \times 10^3$	3.98^A	NA	2.95^C	4.15^A
E. coli	$3.9 \pm 0.6 \times 10^6$	1.48^A	-0.54^D	0.74^B	0.34^C
Enterococcus spp.	$6.3 \pm 0.2 \times 10^6$	0.54^A	< -5.00	0.41^A	0.26^B
S. aureus	$7.9 \pm 0.2 \times 10^5$	0.33^A	-1.43^B	0.50^A	-1.43^B
Salmonella spp.	$2.5 \pm 0.1 \times 10^6$	2.00^A	< -5.00	0.91^C	1.11^B
E. coli O157:H7	$6.4 \pm 0.3 \times 10^6$	0.80^A	-0.71^B	0.66^A	0.67^A

***Notes:** The soluble fraction of influent and effluent were collected as described in the text. A small volume (20 µl) of influent was used as the inoculum for total aerobic, anaerobic, and facultative organisms. The filtrate was inoculated with each test organism and incubated without mixing under anaerobic conditions for 3 days at 28°C. Concentration values are average ± standard deviation (n=3).

NA , not applicable.

$^{A-D}$ Values with the same letter within the same row are not significantly different ($P < 0.05$).

Table 3-4. Growth of indigenous indicator bacteria and *S. aureus* in the whole fraction of wastewater.

Organism	Log_{10} Reduction					
	Influent			Effluent		
	Initial conc, CFU/ml	38°C	28°C	Initial conc, CFU/ml	38°C	28°C
Total anaerobes	$1.3 \pm 0.4 \times 10^7$	$-0.5^{B,C}$	-0.6^{C}	$3.4 \pm 1.1 \times 10^6$	-0.2^{A}	$-0.4^{A,B}$
Total aerobes	$2.1 \pm 0.2 \times 10^7$	-0.2^{A}	-0.1^{A}	$7.9 \pm 1.0 \times 10^6$	-0.1^{A}	-0.6^{B}
Total facultative	$9.4 \pm 0.3 \times 10^6$	-1.0^{B}	-1.4^{C}	$7.6 \pm 0.5 \times 10^6$	-1.0^{B}	-0.4^{A}
Fecal coliforms	$2.4 \pm 0.3 \times 10^5$	< -4.0	< -3.0	$2.8 \pm 0.2 \times 10^4$	< -3.0	< -2.0
Enterococcus spp.	$7.2 \pm 1.2 \times 10^5$	-1.3^{C}	-1.0^{B}	$2.2 \pm 1.2 \times 10^5$	-0.8^{A}	-0.7^{A}
S. aureus	$1.3 \pm 0.6 \times 10^3$	-0.9^{B}	-0.7^{A}	$1.0 \pm 0.1 \times 10^2$	$-0.8^{A,B}$	$-0.8^{A,B}$

[A-C] Values with the same letter in the same row are not significant ($P < 0.05$)

Notes: Influent and effluent samples were incubated with under anaerobic conditions for 3 days at 38°C (mixing) or 28°C (static). Total anaerobes, aerobes, fecal coliforms, *Enterococcus* spp., and *S. aureus* were measured as described in the text. Concentration values are average ± standard deviation (n=3).

Table 3-5. Comparison of growth between indigenous bacteria in wastewater at 38°C and 28°C.

Organism	Log_{10} Reduction			
	Influent		Effluent	
	38°C	28°C	38°C	28°C
Total anaerobes	-0.5^{B}	-0.6^{B}	-0.2^{A}	-0.4^{A}
Total aerobes	-0.2^{A}	-0.1^{A}	-0.1^{A}	-0.6^{B}
Total facultative	-1.0^{C}	-1.4^{D}	-1.0^{B}	-0.4^{A}
Fecal coliforms	< -4.0	< -3.0	< -3.0	< -2.0
Enterococcus spp.	-1.3^{D}	-1.0^{C}	-0.8^{B}	$-0.7^{B,C}$
S. aureus	-0.9^{C}	-0.7^{B}	-0.8^{B}	-0.8^{C}

[A-D] Values with the same letter in the same column are not significant ($P < 0.05$)

Notes: Influent and effluent samples were incubated under anaerobic conditions for 3 days at 38°C (mixing) or 28°C (static). Total anaerobes, aerobes, fecal coliforms, *Enterococcus* spp., and *S. aureus* were measured as described in the text.

Table 3-6. Determination of inhibitory or nutritional limitation on the growth of indigenous *S. aureus*.

Condition	Initial conc, CFU/ml	Final conc, CFU/ml	Log_{10} increase or (decrease)	Log_{10} contribution of 0.09% TSB
0% TSB	$3.4 \pm 0.3 \times 10^4$	$1.3 \pm 0.1 \times 10^3$	-1.4^F	
0.09 % TSB	$6.1 \pm 0.5 \times 10^3$	$1.0 \pm 0.2 \times 10^7$	3.2^A	
Influent	$2.1 \pm 0.4 \times 10^4$	$4.4 \pm 0.4 \times 10^5$	1.3^D	
Influent + 0.09% TSB	$1.7 \pm 0.2 \times 10^4$	$4.3 \pm 0.7 \times 10^6$	2.4^B	1.1^B
Effluent	$1.5 \pm 0.3 \times 10^4$	$2.5 \pm 0.2 \times 10^3$	-0.8^E	
Effluent + 0.09% TSB	$1.6 \pm 0.4 \times 10^4$	$6.4 \pm 1.6 \times 10^5$	1.6^C	2.4^A

Notes: The soluble fraction of influent and effluent were collected by centrifugation at 11,000 x g for 30 mins. The supernatant was filtered using a 0.45 mm nitrocellulose filter. The filtrates were supplemented with tryptic soy broth (TSB) to a final concentration of 0.09%. The filtrates, supplemented filtrates, and 1X PBS (0% TSB) were inoculated with *S. aureus* isolated from influent and incubated without mixing under anaerobic conditions for 3 days at 28°C. Samples were assayed daily for test organisms. Concentration values are average ± standard deviation (n=3).

$^{A-F}$ Values with the same letter within the same column are not significantly different ($P < 0.05$).

Table 3-7. Growth of *S. aureus* in soluble effluent and soluble effluent with various amendments.

Condition	Initial conc, CFU/ml	Final conc, CFU/ml	Log increase or decrease
1X PBS	$1.1 \pm 0.15 \times 10^4$	$5.2 \pm 1.2 \times 10^3$	-0.5^G
3% TSB	$9.7 \pm 1.5 \times 10^3$	$5.4 \pm 1.0 \times 10^7$	3.3^B
Influent	$1.2 \pm 0.25 \times 10^4$	$3.1 \pm 0.38 \times 10^7$	2.4^D
Effluent	$7.0 \pm 2.0 \times 10^3$	$2.8 \pm 0.3 \times 10^3$	-0.4^G
Glucose, 5 mM	$9.7 \pm 1.2 \times 10^3$	$4.8 \pm 3.2 \times 10^2$	-1.4^H
Effluent + glucose, 5 mM	$9.7 \pm 1.2 \times 10^3$	$7.7 \pm 1.5 \times 10^4$	0.9^E
Glucose, 55 mM	$6.0 \pm 1.3 \times 10^3$	$2.2 \pm 0.53 \times 10^3$	-0.4^G
Effluent + glucose, 55 mM	$1.6 \pm 0.31 \times 10^4$	$9.5 \pm 0.33 \times 10^7$	3.6^A
Yeast Extract, 0.1%*	$2.6 \pm 0.36 \times 10^4$	$2.3 \pm 0.93 \times 10^7$	2.5^C
Effluent + Yeast Extract, 0.1%	$2.9 \pm 0.15 \times 10^4$	$2.4 \pm 0.66 \times 10^7$	2.9^C
Ashed Yeast Extract	$3.2 \pm 0.7 \times 10^4$	$1.6 \pm 0.2 \times 10^4$	-0.3^G

*Yeast extract at 0.1% is equivalent to 5 mM glucose on a carbon basis (*see* Table 3-1).

Notes: Phosphate buffered saline (1X PBS), 3% tryptic soy broth (TSB), influent, effluent, supplemented effluent, and supplement solutions in sterile saline were inoculated with *S. aureus* suspended in PBS. Samples were incubated without mixing under anaerobic conditions for 3 days at 28°C. Concentration values are average ± standard deviation (n=3).

$^{A-H}$ Values with the same letters within the same column are not significantly different ($P < 0.05$).

CHAPTER 4
INDICATOR AND PATHOGENIC BACTERIA REDUCTION DURING ANAEROBIC DIGESTION: ATTACHMENT TO THE FIXED FILM

Introduction

Fixed-bed anaerobic digesters are systems that use internal support media where anaerobic bacteria attach to the surface and proliferate, resulting in the formation of a biofilm (*see* Chapter 2). The retained biofilm (i.e. fixed-film) allows for a higher treatment capacity as compared to systems that do not use internal support media (Ramasamy and Abbasi, 2000). The increased treatment capacity for systems with internal support media is due to the retention of methanogens, which grow at a slow rate (Wilkie and Colleran, 1989). Digesters that are not constructed with an internal support media can potentially lose a substantial portion of the methanogenic population as effluent exits the system. To maintain treatment efficiency, such systems have to operate at long retention times (> 10 days) to reestablish a methanogenic population.

Pathogen reduction by anaerobic digestion has been well studied in various systems, including a fixed-film system (Barnes, 2002, Davis et al., 2001, Hill, 2003). However, for fixed-film systems, the role of the retained biofilm in pathogen reduction during anaerobic digestion remains unclear.

Purpose

The purpose of the current study was to use a model organism to determine if indicator and pathogenic bacteria were removed from the liquid phase during anaerobic digestion by attachment to the fixed-film.

Materials and Methods

Pilot-Scale Fixed-Film Reactors

The two pilot-scale fixed-film reactors used for biofilm attachment studies had a total capacity of 104 gal each. Both reactors were operated at ambient temperature (28°C to 33°C) and a 3-day HRT. The systems were considered at steady state once SCOD reduction was consistent (69% ± 5%). Both reactors were packed with vertically orientated PVC pipes to provide internal support media for biofilm formation. Each system was continuously fed influent from a feeding manifold by a peristaltic pump (MasterFlex®). However, one unit was operated in up-flow mode, where contents entered the reactor from the bottom and the other was operated in down-flow mode, where contents entered the reactor from the top. Contents of both reactors were mixed, by recycling at a 3:1 ratio to feed rate, by another peristaltic pump.

Bacterial Culture

Escherichia coli BL(21)DE3 haboring plasmid plAM1055, for the production of green fluorescent protein (GFP), was grown at 37°C overnight in LB broth, Lennox (Fisher) with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Amresco®, Solon, OH) and 15.4 μg/ml of ampicillin (Sigma). The cells were harvested by centrifugation at 2,000 x g for 10 minutes and washed three times with sterile 1X PBS. The cells were resuspended in 100 ml 1X PBS and stored at 5°C until use.

Survival Comparability with Indigenous Fecal Coliforms

Influent and effluent samples were collected as previously described (*see* Chapter 3). GFP producing *E. coli* were inoculated into whole fractions of influent and effluent. Samples were incubated under anaerobic conditions (*see* Chapter 3) for 3 days at 28°C. Indigenous fecal coliforms were assayed daily as previously described (*see* Chapter 3).

GFP producing *E. coli* were assayed daily using plate count agar supplemented with 0.00002% crystal violet (Carolina Biological Supply Co., Burlington, NC), 0.4 mM IPTG, and 15.4 μg/ml ampicillin. Following incubation, GFP producing *E. coli* colonies were enumerated by exposing the plates to a UV light source (366 nm).

Biofilm Attachment Studies

A 100 ml suspension of GFP producing *E. coli* (1.2×10^7 CFU/ml) was prepared as previously described. The suspension of bacteria was fed into the reactor through the feeding pumps. Effluent samples from the reactor were collected at 1 h intervals over 12 h, and then a composite sample was taken daily. Serial dilutions of effluent samples were done using 1X PBS. Viable counts of GFP producing *E. coli* were performed by direct plating diluted samples onto plate count agar prepared and incubated as previously described. Total counts of GFP producing *E. coli* were performed by filtering diluted samples through a 0.45 μm polycarbonate filter (47 mm, Nucleopore Corp., Pleasanton, CA). Filters were mounted on glass slides (50 mm x 75 mm) and cells were enumerated under 250X total magnification with a fluorescent microscope (Carl Zeiss, Standard 25) equipped with a UV light source (470 nm). The total number of test organisms expected to be present in the effluent at each sampling event was calculated by the following:

Predicted effluent volume = (reactor volume / HRT) x sampling time interval

Predicted effluent CFU = (initial CFU / reactor volume) x predicted effluent volume

Biofilm Sampling and Examining

Support media in close proximity to where influent entered each system were chosen for sampling. Biofilm samples were obtained by removing selected support media from each reactor. Then, 2 in sections were cut from the bottom (up-flow reactor) and top (down-flow reactor) of the support media. Each 2 in section was then divided into

smaller sections and placed inside containers lined with moist paper towels to prevent desiccation. The sections were examined under 300X total magnification with a stereomicroscope (Leica MZ7s) equipped with a UV light source (470 nm). Photos of the samples were taken using a digital camera (Nikon Coolpix 4500) connected to the microscope by a lens attachment (Martin Microscope mmcool, S/N: 0941).

Results

Fecal Coliform and GFP *E. coli* Survival

The GFP producing *E. coli* displayed comparable survival with indigenous fecal coliforms in both the influent and effluent, suggesting that the test organism could serve as a suitable model for indigenous fecal coliforms (Figure 4-1).

Biofilm Attachment Studies

Each pilot-scale fixed-film reactor was fed a suspension of GFP producing *E. coli*. The number of cells detected at each sampling event in the up-flow reactor was lower than the predicted values (Figure 4-2). There was a 1 $\log_{10}$ difference between the predicted values and the experimental values at each sampling event. Also, the model organism could not be detected on Day 4 and after Day 6. After 1 HRT (3 days), there was a 0.9 $\log_{10}$ reduction of the model organism.

During the first 12 h, retention of the model organism in the down-flow reactor was similar to the upflow-reactor (Figure 4-3). However, on Day 1, 4 and 6, the experimental values were similar to the predicted values. The model organism was not detected after Day 7. After 1 HRT, there was a 0.2 $\log_{10}$ reduction of the model organism.

To confirm the attachment of the model organism to the fixed-film, biofilm samples were obtained and examined. GFP producing *E. coli* were detected on the fixed-film (Figure 4-4). However, the viability of the attached organisms was not determined.

Discussion and Conclusions

The current study demonstrates the role of the fixed-film for reducing indicator and pathogenic bacteria during digestion. Our results show that the test organism attached to the fixed-film, thereby reducing effluent concentrations of the test organism. However, a difference in retention was observed between the two modes of operation. After 3 days of operation, the reduction in the up-flow mode (0.7 $\log_{10}$) was higher than in the down-flow mode (0.1 $\log_{10}$). Conversely, Barnes (2002) reported a 0.2 $\log_{10}$ reduction of fecal coliforms, using the same reactor operated in up-flow mode at the same conditions as the current study. Also, in the study, a similar reduction (0.4 $\log_{10}$) of fecal coliforms was achieved during operation in down-flow mode. However, in the current study, the difference between the two modes suggests that operation in up-flow mode may lead to more interaction between the fixed-film and indicator and pathogenic organisms.

Anaerobic systems that employ fixed-beds have demonstrated higher treatment efficiencies than systems without fixed-beds. However, the contribution of the fixed-film to pathogen reduction from various substrates has not been extensively studied. The current study provided evidence that the fixed-film contributes to the reduction of indicator and pathogenic bacteria during anaerobic digestion. After 3 days of operation, a 0.7 $\log_{10}$ reduction was achieved. Previously, microbial competition was shown to account for more than 2 $\log_{10}$ reduction of indicator bacteria in batch studies (*see* Chapter 3). However, the attachment studies were performed with a continuously fed system, where substrates and organisms were constantly introduced. In continuously fed systems, microbial competition may still have a significant contribution to indicator and pathogen reduction, but the constant influx of substrates and organisms may decrease this effect, in comparison to the batch studies. However, in the current study, model organisms

detected in the effluent were not viable. This result suggests that inactivation of the model organism occurred in the liquid phase during digestion. Previous results (*see* Chapter 3) suggest that the inactivation in the liquid phase observed in the current study may be due to microbial competition. In a continuously fed system, the fixed-film provides a physical means for removal, whereas microbial competition may inactivate unattached organisms in the liquid phase. The combination of attachment to the fixed-film and microbial competition may synergistically contribute to the reduction of indicator and pathogenic bacteria during digestion in the fixed-film reactor.

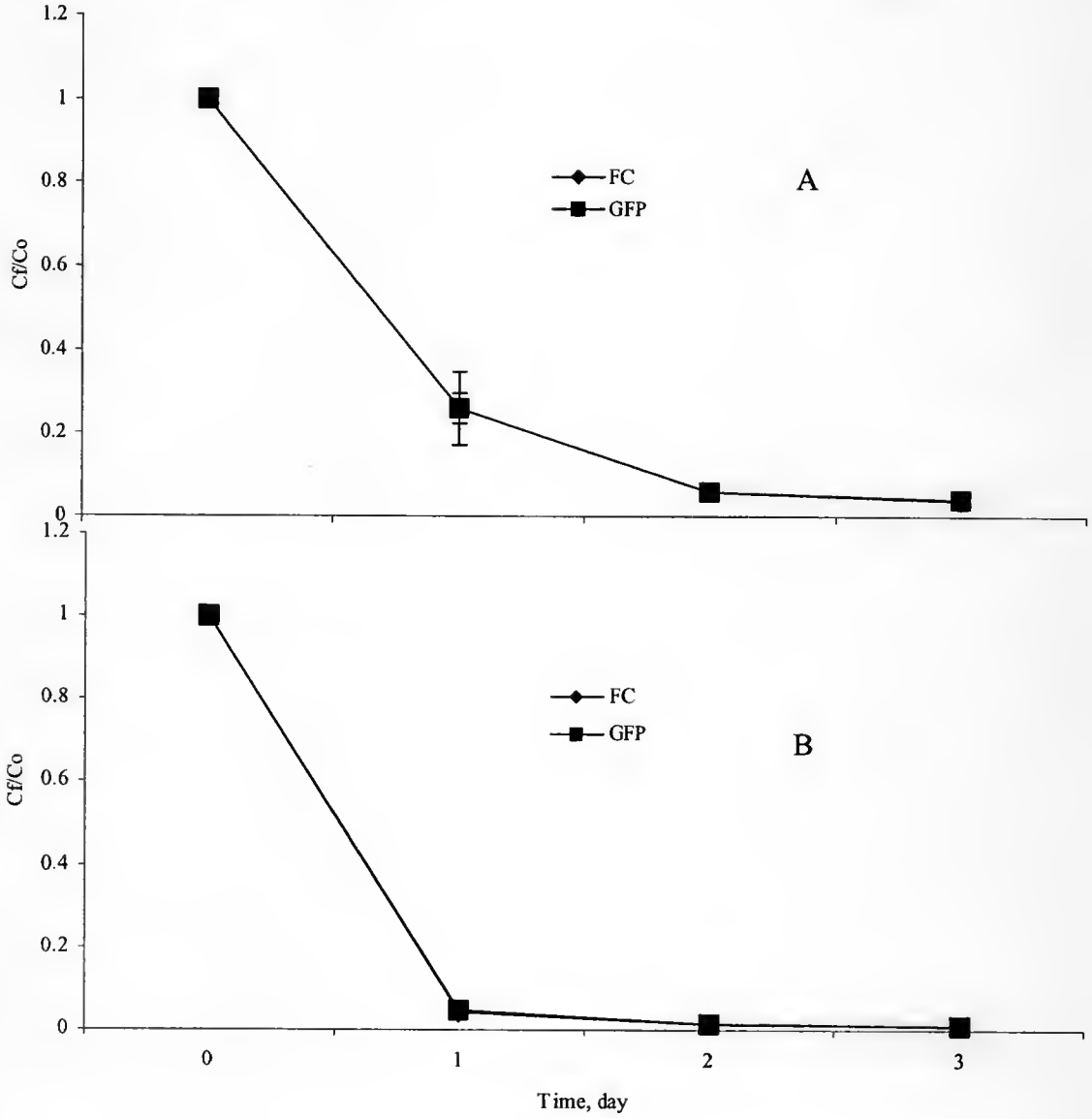

Figure 4-1. Survival of indigenous fecal coliforms and GFP producing *E. coli* in wastewater.

Notes: Influent (A) and effluent (B) were inoculated with GFP producing *E. coli* suspended in PBS. Samples were incubated under anaerobic conditions for 3 days at 28°C. Fecal coliforms (□) and GFP producing *E. coli* (□) were assayed daily as described in the text. Each point is an average and error bars represent standard deviation (n=3).

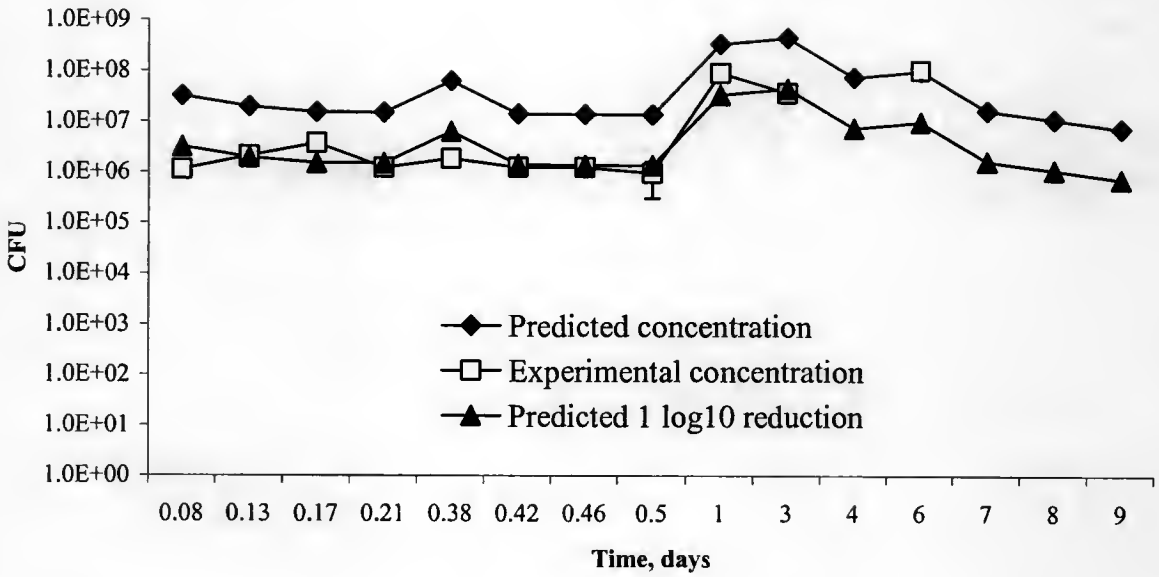

Figure 4-2. Retention of GFP *E. coli* within the pilot-scale fixed-film anaerobic digester operated in up-flow mode.

Notes: A pilot-scale fixed-film reactor was fed a suspension of GFP producing *E. coli*. Effluent samples were collected and total counts of GFP producing *E. coli* were performed as described in the text. Each point represents triplicate samples and error bars represent standard deviation.

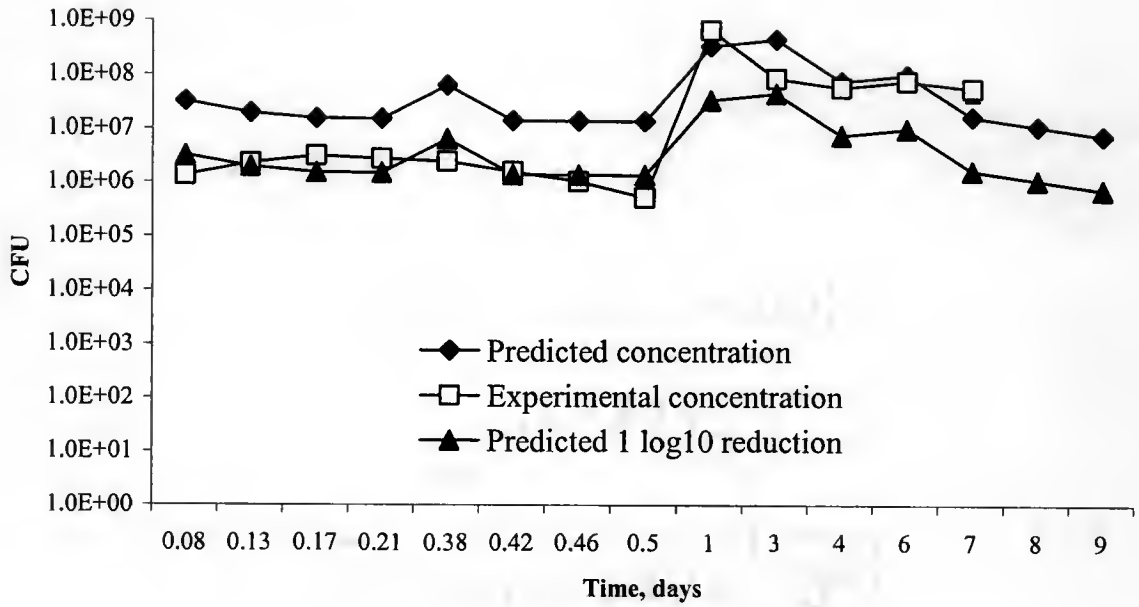

Figure 4-3. Retention of GFP producing *E. coli* within the pilot-scale fixed-film operated in down-flow mode.

Notes: A pilot-scale fixed-film reactor was fed a suspension of GFP producing *E. coli*. Effluent samples were collected and total counts of GFP producing *E. coli* were performed as described in the text. Each point represents triplicate samples and error bars represent standard deviation.

Figure 4-4. GFP producing *E. coli* attached to the surface of the biofilm.

Notes: Samples of internal support media were removed from the pilot-scale fixed-film reactor. Samples were cut from support media and viewed under 300 X total magnification with a UV light source (470 nm). White arrows indicate GFP producing E. coli.

CHAPTER 5
BACTERIOPHAGE REDUCTION DURING ANAEROBIC DIGESTION: THE ROLE OF INDIGENOUS MICROFLORA

Introduction

Animal manure may contain pathogenic viruses that pose severe human and herd health concerns (Bicudo and Goyal, 2003, Pell, 1996). Therefore, treatment of manure is necessary to reduce the levels of these organisms. Anaerobic digestion can effectively reduce the concentration of viruses from various substrates, including manure. Berg and Berman (1980) found that mesophilic and thermophilic anaerobic digestion (20 day HRT) of domestic sludges achieved a 1 $\log_{10}$ and 3 $\log_{10}$ reduction, respectively, of viruses after 20 days. Similarly, Huyard et al. (2000) investigated virus reduction in sludge by a two-phase anaerobic digester (TPAD) operated at thermophilic (2 day HRT) and mesophilic (10 day HRT) temperature. The authors reported a 4 $\log_{10}$ reduction of poliovirus after 10 days. Also, Aitken et al. (2005) demonstrated that thermophilic anaerobic digestion, at a 4 day HRT and treating sludge, achieved > 4 $\log_{10}$ reduction of poliovirus. However, reduction of male-specific bacteriophages was 1 to 2.6 $\log_{10}$. Spillman et al. (1987) reported inactivation rates of less than 1 $\log_{10}$ day^{-1} for viruses during mesophilic anaerobic digestion of sludge. Inactivation rates during thermophilic digestion varied between 0.2 to > 8 $\log_{10}$ hr^{-1}. However, the authors did not report the HRT of the digester used in the study.

Virus reductions similar to the aforementioned studies have been found during treatment of animal manure. Lund et al. (1996) found that mesophilic anaerobic

digestion (15 day HRT) of mixed animal manure (25% pig, 75% cow) achieved a 4 $\log_{10}$ reduction of bovine enterovirus and porcine parvovirus after 1 and 2 days, respectively. Derbyshire et al. (1986) examined the occurrence of viruses in pig manure following mesophilic anaerobic digestion. The authors detected viruses during the first 5 months of the study. However, the authors did not report virus concentrations, virus reduction rates, nor the HRT of the digester. Treatment of dairy manure wastewater by fixed-film anaerobic digestion at a 3 day HRT and ambient temperature (28°C), can achieve a 1 $\log_{10}$ reduction of bacteriophages and viruses (Barnes, 2002, Davis, 2001).

Previous studies on viral inactivation by anaerobic digestion point reductions to operational parameters (i.e., temperature and retention time). However, antagonism by indigenous manure microflora has been suggested to play a critical role in inactivating viruses during anaerobic digestion (Deng and Cliver, 1995, Ward, 1982). Therefore, the role of indigenous microflora in reducing virus during anaerobic digestion should be investigated.

Purpose

The purpose of the current study was to determine if the reduction of viruses in flushed dairy manure wastewater during fixed-film anaerobic digestion at ambient temperature is attributed to the presence of indigenous manure microflora.

Materials and Methods

Sample Collection, Characterization, and Preparation

Flushed dairy manure wastewater (influent) and anaerobically treated flushed dairy manure wastewater (effluent) were collected from the University of Florida Dairy Research Unit (DRU) in Hague, FL and characterized as previously described (*see* Chapter 3)

Bacteriophages

Bacteriophages and their respective hosts were MS2 (ATCC 15597-B1) and *E. coli* C-3000 (ATCC 15597), ΦX174 (ATCC 13706-B5) and *E. coli* (ATCC 13706), and PRD1 and *Salmonella* Typhimurium (ATCC 19585).

Experimental Design

Whole and soluble fractions of influent and effluent were prepared as previously described (*see* Chapter 3). Soluble fractions were inoculated with each organism. The samples were transferred to the anaerobic chamber and incubated for 3 days at 28°C. Groundwater collected from the DRU was used as a negative control for experiments using bacteriophages.

Bacteriophage Quantification

Bacteriophages were enumerated as plaque forming units per ml (PFU/ml) using the soft agar overlay technique (Hurst, 1997).

Anaerobic Conditions

Anaerobic conditions during incubation were maintained in an anaerobic chamber as previously described (*see* Chapter 3).

Statistical Analysis

Log_{10} increases or decreases were determined under each condition by subtracting the final log_{10} transformed concentration from the initial log_{10} transformed concentration. Each condition was compared with the positive control and negative control using one-way ANOVA and Duncan's Method with a significance level of 0.05. The statistical analysis was performed using ProStat© v 3.5.

Results

Wastewater Characteristics

Characteristics of the wastewaters used for experiments are given in Chapter 3 (*see* Table 3-2).

Influence of Whole and Soluble Fractions of Wastewater

To determine if the indigenous microflora affected the concentrations of bacteriophages, influent and effluent with and without indigenous microflora (i.e., whole and soluble fractions, respectively) were inoculated with approximately 10^5 PFU/ml of bacteriophages. The samples were incubated under anaerobic conditions for 3 days at 28°C. In the soluble fractions of influent and effluent, bacteriophages (MS2, PRD1 and ΦX174) were stable throughout the duration of the experiment, and were not significantly different ($P > 0.05$) from those observed in groundwater (Table 5-1). However, when incubated in the presence of indigenous microflora, there were significant reductions ($P < 0.05$) in the concentrations of each bacteriophage in both influent and effluent (Table 5-1). There was a significantly higher reduction ($P < 0.05$) of MS2 and ΦX174 in effluent than in influent. The highest reductions were observed with PRD-1. However the reductions of PRD1 observed in influent and effluent were not significantly different ($P < 0.05$). These results show that the activity of indigenous microflora in influent and effluent can contribute to bacteriophage inactivation under anaerobic conditions and ambient temperature.

Discussion and Conclusions

The current study demonstrates the role of indigenous microflora on inactivation of viruses during anaerobic digestion. All viruses studied were stable in soluble fractions of wastewater incubated under anaerobic conditions at 28°C for 3 days. However, in the

presence of indigenous microflora, all the viruses studied were reduced by greater than 1 $\log_{10}$.

Previous studies have shown inactivation of viruses during mesophilic and submesophilic (15 to 25°C) temperatures may be attributed by microbial antagonism. Pesaro et al. (1995) conducted field studies to investigate virus inactivation in liquid cattle manure stored under anaerobic conditions using membrane sandwiches with and without pores. At submesophilic temperatures, decimation times for viruses were significantly lower for membranes with pores than those without. The results suggest that microbial products may contribute to inactivation of viruses. Ward (1982) investigated antiviral activity of microorganisms in activated sludge (mixed-liquor suspended solids – MLSS) against poliovirus 1 under aerobic conditions. The study demonstrated that viruses were inactivated in the presence of MLSS and in broth previously incubated with MLSS. These results suggested that microbial products could contribute to inactivation of viruses during anaerobic treatment. Deng and Cliver (1992) found similar inactivation of poliovirus 1 by bacteria in unmixed, stored swine manure. The authors reported that decimation times in manure incubated under aerobic conditions at 37°C and 25°C were significantly less than those in cell-free controls at the same temperatures. A later study by the same authors suggest that microbial mediated inactivation of viruses may not always be enzymatic (Deng and Cliver, 1995). Production of antiviral substances that are not enzymatic (virolytic substances) has been demonstrated with *Pseudomonas aeruginosa* (Cliver and Herrmann, 1972).

The current study provides further evidence of the critical role indigenous microflora have during fixed-film anaerobic digestion. Although microbial processes

during anaerobic digestion are shown to reduce virus concentrations, complete removal is not achieved. Reductions of greater than 3 $\log_{10}$ may be achieved, but as demonstrated by previous studies either longer retention times or higher operating temperatures are necessary to allow for more reduction. However, operations desiring to minimize energy input to digesters must balance performance with wastewater production, thereby sacrificing extensive virus removal. Therefore, effluents from digesters will inherently contain residual concentrations of active viruses, which may be introduced to the environment during land application.

Table 5-1 Impact of indigenous microflora on viruses suspended in groundwater and wastewater.

Organism	Log_{10} Reduction					
	Groundwater		Influent		Effluent	
	Soluble fraction	Whole fraction	Soluble fraction	Whole fraction	Soluble fraction	Whole fraction
MS2	-0.5^{B}	-0.5^{a}	-0.2^{A}	-0.8^{b}	0.0	-1.3^{b}
PRD1	-0.3^{A}	-0.3^{a}	-0.1^{A}	-2.5^{b}	-0.2^{A}	-2.4^{b}
ΦX174	-0.5^{A}	-0.5^{a}	-0.2^{A}	-1.2^{b}	-0.3^{A}	-1.8^{c}

Notes: Groundwater, influent, and effluent were prepared as described in the text (*see* Chapter 3). Samples were inoculated with viruses and incubated under anaerobic conditions at 28°C for 3 days. Each value is an average log_{10} reduction (n = 3).

$^{A-B, a-c}$ Values with the same letter within the same row are not significantly different ($P < 0.05$).

CHAPTER 6
TRANSPORT OF VIRUSES IN SOIL AMENDED WITH ANAEROBICALLY DIGESTED FLUHSHED DAIRY MANURE WASTEWATER

Introduction

Intensification and concentration of large dairy operations have led to issues with manure management. Two major concerns are nuisance odors and possible contamination of groundwater resources following land application of manure. In an attempt to reduce these problems, a fixed-film anaerobic digester has been constructed at the University of Florida's Dairy Research Unit (DRU). Freestall barns at the DRU are hydraulically flushed with water to remove animal manure. The fixed-film digester then treats the flushed dairy manure wastewater (influent) before it is land applied for forage crop production. Anaerobic digestion significantly reduces the COD of flushed dairy manure (Wilkie, 2005). Previous studies have shown that this unit can also reduce the levels of indicator and pathogenic bacteria and bacteriophages by approximately 90% (Davis et al., 2001). However, the use of fixed-film anaerobic digesters on flushed manure is a relatively new technology and the fate of viruses following land application of anaerobically digested flushed dairy manure (effluent) is unknown.

Virus Inactivation during Anaerobic Digestion

Viruses are known to be associated with and survive in animal manure (Elliott and Ellis, 1977, Mawdsley et al., 1995, Pesaro et al., 1995). However, anaerobic treatment has been shown to inactivate viruses. Anaerobic digestion at ambient temperature (25 to 31°C) and a 3-day HRT has been shown to achieve a 1.5 $\log_{10}$ reduction of somatic and

male-specific bacteriophages from flushed dairy manure wastewater (Davis et al., 2001). Lund et al.(1996) found that mesophilic (35°C) anaerobic digestion of swine and cow liquid manure required 9 days to achieve a 4 $\log_{10}$ reduction (99.99%). Under thermophilic (55°C) conditions, the system required 6 days to achieve a 4 $\log_{10}$ reduction of porcine parvovirus. Anaerobic digestion is effective for reducing the load of viruses in animal manure before land application. However, residual viral concentrations can be detected in effluents of anaerobic systems treating animal manure and be detected in soil where wastewater has been applied (Derbyshire, 1976, Derbyshire et al., 1986) Although anaerobic digestion achieves significant reductions of viruses from animal manure, residual concentrations of viruses can potentially lead to groundwater contamination following land application of effluents.

Viral Attachment to Soil

Viruses in water utilize two mechanisms for attaching to soil, hydrophobic and electrostatic interactions. Physical and chemical parameters that affect virus adsorption to soil include the pH of the aqueous media and the soil, flowrate through the soil matrix, soil organic matter, percent clay, and ionic strength of the suspending medium (Goyal and Gerba, 1979, Kinoshita et al, 1993, Lance et al., 1976, Zhuang and Jin, 2003).

pH and isoelectric point. Previous studies have investigated transport, adsorption, and survival of viruses in wastewater following land application of wastewater. Several environmental factors have been found to influence viral adsorption in soil. Organic matter and exchangeable iron are inversely correlated with viral adsorption to soil (Gerba and Goyal, 1981, Hurst et al., 1980). However, one of the most important factors is pH (Goyal and Gerba, 1979). In general, an increase in soil pH will result in a decrease in viral adsorption, whereas a decrease in soil pH will increase viral

adsorption. However, environmental factors, including pH, do not influence the adsorption of all viruses to the same degree. Gerba and Goyal (1981) examined the differences in adsorption among viruses and categorized the viruses into two groups based on factors that contributed to adsorption. The factors that were most important for adsorption of group I viruses, which included ΦX174 and MS2, to soil were pH, organic matter content, and exchangeable iron. However, group II viruses, which included poliovirus 1 LSc and coxsackie B3, soil particle surface area was the only significant variable that contributed to adsorption.

The impact of pH on viral adsorption is related to the isoelectric point (pI) of the virus (Dowd et al., 1998). The isoelectric point is the pH at which the virus has no net electrical charge. In general, viral adsorption will occur in soils with a pH lower than that of the pI of virus. Viruses and soil particles generally have a net negative charge at a neutral pH. At low pH values, viruses acquire a net positive charge, resulting in adsorption to soil. At high pH values, viruses acquire a net negative charge and adsorption to soil is minimal and results in elution or desorption of the virus from the soil. The adsorption effects observed with viruses are the result of alterations to the proteins on the surface of the virion during pH changes; whereas changes to the charge on soil particles are minimal (Berg, 1987, Dowd et al., 1998).

Hydrophobic interactions. The side chains of several amino acids found on the protein coat of viruses are nonpolar and attract other nonpolar compounds or groups. Hydrophobic bonding occurs when nonpolar groups aggregate. Viruses with coats that are predominately composed of hydrophobic proteins will tend to use hydrophobic interactions. Such viruses include MS2 and echovirus 5 (Shields and Farrah, 2002).

Futhermore, viruses that contain host-derived phospholipid membranes, such as PRD1, will also utilize hydrophobic bonding (Hanninen et al., 1997). Hydrophobic interactions may be demonstrated when the pH of an aqueous medium is adjusted to the pI of the virus. At that point, hydrophobic interactions may predominate (Berg, 1987).

Electrostatic interactions. Electrostatic interactions occur between charged side chains of amino acids on viral protein coats and charges on surfaces. Shields and Farrah (2002) compared viral adsorption by electrostatic and hydrophobic interactions. Using DEAE-Sepharose, a solid with charged groups, virus adsorption was characterized by elution from the solids with solutions increasing in ionic strength. Several viruses, including poliovirus 1 LSc and T4, were shown to utilize strong electrostatic interactions. In aqueous media, such as natural waters, the pH is usually neutral and above the pI of most viruses. In such instances, the charge of the viron and soil matrix will be the same and electrostatic interactions will depend on the concentration of cations present with in the aqueous media or the soil. Cations may interact with both the viron and soil surface generating a salt bridge, resulting in adsorption (Berg, 1987).

Purpose

Viruses have been shown to be inactivated during anaerobic digestion (Davis et al., 2001, Lund et al., 1996). However, residual concentrations of viruses may be introduced to the environment during land application of treated wastewater. To date, the adsorption of viruses to soil following land application of effluent from a fixed-film anaerobic digester treating flushed dairy manure wastewater has not been studied. The purpose of the current study is to investigate the adsorption and survival of viruses in flushed dairy manure wastewater and in anaerobically digested wastewater to soil.

Materials and Methods

Collection and Analysis of Soil Samples

Soil was collected from the DRU in Hague, FL, in an area mapped as typic Quartzipsamments; sandy soil with greater than 90% having particle size between 0.02 to 2.0 mm (USDA, 1999). Samples were taken at 0.8 m depth from a sprayfield that receives effluent from a tertiary short-term storage pond. Soil samples were thoroughly mixed to yield a composite sample. The soil was air dried at room temperature and sieved. Batch adsorption studies were performed on soil with particle size less than 0.2 mm. To increase the flow rate through columns, soil with particle size of 0.2 to 0.8 mm was used for column studies. Soil pH and conductivity were measured as described by Mylavarapu and Kennelley (2002). Soil particle size analysis was conducted on the whole, batch, and column soil samples.

Collection and Analysis of Wastewater, Groundwater, and Rainwater Samples

One liter samples of influent and effluent were collected from the DRU manure management facility. Grab samples of influent were collected from a wet well prior to anaerobic treatment. Effluent was collected from a port located on the fixed-film anaerobic digester. After settling, supernatant fractions of both wastewaters were used for experiments. Groundwater was collected from wells located at the DRU and rainwater was collected during storm events. Sample pH and conductivity were measured according to standard methods (Standard Methods, 1998).

Viruses and Viral Assays

Bacteriophages and their hosts used in this study were MS2 (ATCC 15597-B1), host *E. coli* C-3000 (ATCC 15597); ΦX174 (ATCC 13706-B5), host *E. coli* (ATCC 13706); and PRD1, host *Salmonella* Typhimurium (ATCC 19585). Phages were

quantified as plaque-forming units (PFU) using the agar overlay technique, as described by Hurst (1997). Poliovirus 1 LSc (ATCC VR-59) was assayed as PFU on Buffalo green monkey kidney cells, as described by Hurst (1997). Characteristics of the viruses used in the study are given in Table 6-1.

Virus Stability

The stability of viruses in groundwater and wastewater was tested. During all batch and column studies, viruses were suspended in groundwater and wastewater and assayed at the beginning and end of each experiment to check for inactivation during an experiment. Viral assays were performed as previously described.

Attachment and Detachment Studies

Batch studies. Batch adsorption studies were performed with groundwater, influent wastewater, and effluent from the anaerobic digester. The study was designed using nine batches so that each experimental condition was performed in triplicate. The entire experiment was performed twice to yield a total of six observations for each experimental condition. Two ml samples of groundwater, influent, or effluent were inoculated with viruses and added to 1 g soil samples (particle size < 0.2 mm). Samples were mixed on a reciprocating shaker at 55 rpm for 1 h, followed by centrifugation at 3,000 x g for 10 min. The supernatant fraction was collected and assayed. The soil pellets were mixed with 10 ml 3% beef extract (BE), pH 7, for 30 min to recover adsorbed viruses (Hurst, 1997). The samples were centrifuged again and the supernatant fraction was collected and assayed for viruses as previously described.

To determine the influence of groundwater, influent, and effluent on desorption of enteroviruses and bacteriophages from soil, 1 g soil samples were set up as previously described. Each soil sample was mixed with 2 ml of inoculated groundwater for 1 h and

centrifuged as previously described. The supernatant fraction was assayed and the soil pellets were treated with 10 ml of either groundwater, 3% BE, influent, or effluent for 30 min. The samples were centrifuged at 3,000 x g for 10 minutes and the supernatant fractions were assayed

Column studies. Adsorption studies with soil columns were performed using 45 g of soil (0.2 to 0.8 mm) packed in 60 ml syringes with a borosilicate fiberglass filter in the bottom of the syringe. Each experimental condition was performed in triplicate. One pore volume (20 ml) of groundwater, influent, or effluent was inoculated with viruses and passed through the columns at 1 ml/min. The percolate was collected and assayed to determine the percentage of viral adsorption. Each column was then removed from the syringes and placed in a 500 ml centrifuge bottle. From each column, 5 g was removed, dried and then mixed with 10 ml of deionized water for 2 h to measure soil pH. The remaining 40 g of soil was mixed for 30 min with 40 ml 10% buffered BE (100 g/L BE, 13.4 g/L sodium phosphate dibasic, 1.2 g/L citric acid) (Hurst, 1997). The samples were centrifuged at 5,000 x g for 10 min and then the supernatant fraction was collected and assayed.

To study potential mobilization of viruses by influent and effluent, one pore volume of groundwater inoculated with viruses was added to each column. The percolate was collected and assayed to determine the percentage of viral adsorption. Six pore volumes of rainwater was added to each column. The percolates were collected and assayed for viruses. An additional six pore volumes of either rainwater, influent, or effluent was passed through the columns. The percolates were collected and assayed for

viruses. The columns were removed from the syringes and mixed with BE and assayed for recovered viruses as described previously.

Attachment and Detachment Mechanisms

Soil COD retention. The amount of soluble organics retained by the soil, expressed as soil soluble chemical oxygen demand (soil SCOD), was calculated by subtracting wastewater initial SCOD from SCOD of the wastewater after percolating through the soil column. The percentage of viruses mobilized at each pore volume was plotted against soil-retained SCOD for the corresponding pore volume and analyzed by linear regression.

Influence of pH on virus adsorption. A 100 ml sample of either groundwater, influent, or effluent was adjusted to pH 3.5 with HCl. Phages were added to the adjusted groundwater and wastewater samples. Inoculated samples (2 ml) were added to 1 g of soil samples (< 0.2 mm) and mixed on a reciprocating shaker at 55 rpm for 1 h. The samples were centrifuged, assayed, and the viruses were recovered as described previously for batch studies.

Wastewater fractionation. Influent and effluent samples were fractionated using cellulose ester dialysis tubing (Spectra/Por® Biotech). Groundwater, influent, and effluent samples (20 ml) were transferred to dialysis tubing with molecular weight cut-offs (MWCOs) of 100 kDa, 10 kDa, and 1 kDa. Groundwater samples were dialyzed against 1 L influent for 3 days at 4°C. Influent and effluent samples were dialyzed against 2 L deionized water for 3 days at 4°C. A larger volume of deionzed water was used for dialyzing wastewater samples to remove as much of the target sized compounds as possible. The samples were removed and a 10 ml aliquot of each sample was

inoculated with viruses. Adsorption to soil, virus assays, and virus recovery was performed as previously mentioned for batch studies.

Influence of detergents on adsorption and desorption. Adsorption studies were performed using a cationic detergent (hexadecyltrimethylammonium bromide, HTAB, Sigma®), anionic detergent (sodium dodecyl sulfate, SDS, FisherBiotech) and a nonionic detergent (polyoxyethylene sorbitan monooleate, Tween 80, Fisher Scientific) were added to groundwater, influent, and effluent samples to a final concentration of 0.01%. The samples were inoculated with viruses and adsorption, assay, and recovery was performed as previously described for batch studies.

Batch soil samples (1 g, particle size < 0.2 mm) were pretreated with groundwater supplemented with HTAB, SDS, or Tween 80 to a final concentration of 0.01% to determine the impact on virus adsorption to soil. Batch samples were mixed with 10 ml of groundwater detergent solution for 1 h on a reciprocating shaker table as previously mentioned. The samples were centrifuged as described earlier for batch studies and the supernatant was discarded. The pellet was washed three times with 10 ml groundwater to remove any detergent not retained by the soil. To the final soil pellets, 2 ml of groundwater inoculated with viruses was added. The samples were mixed, centrifuged, assayed, and the viruses were recovered as previously described for batch studies.

The influence of detergents on desorption of viruses attached to soil samples was performed by adsorbing viruses in groundwater to soil as previously described for batch soil adsorption studies. Groundwater was supplemented with HTAB, SDS, or Tween 80 to a final concentration 0.01%. The soil samples with attached viruses were mixed with 10 ml of detergent solution for 30 min, centrifuged, and assayed as previously described

for batch soil studies. A secondary elution was performed with 3% BE as previously described for batch soil studies.

Virus Survival in Soil

The survival of viruses in groundwater and wastewater attached to soil was determined. Groundwater, influent, and effluent were inoculated with MS2, PRD1, ΦX174, and poliovirus 1 LSc at 1 x 10^7 PFU/ml. Inoculated solutions were added to soil as previously described for batch soil studies. Samples were set up in triplicate for each time point. Once a week, viruses were recovered with 3% BE and assayed as previously described for batch soil studies.

Risk Assessment of Flushed Dairy Manure

A risk assessment was performed to determine the probability of infection following exposure to groundwater where influent and effluent was applied to land. The following exponential dose-response model was used to estimate the risk of infection:

$$\pi = 1 - 10^{-(d/k)}$$

where, π is the probability of infection, d is the amount of exposure to the organism, and k is the infective dose (Rose and Hass, 1999). Infectious dose data for adenovirus (PHAC, 1999), rotavirus (Graham *et al*, 1987), and Norwalk virus (Schaub and Oshiro, 2000) was used for the risk assessment.

Statistical Analysis

The percentage of viruses eluted was calculated by dividing the amount of viruses in the eluent by the amount of viruses adsorbed to soil. The mean percentages of viruses eluted and the differences in viral adsorption between natural and detergent-pretreated conditions were analyzed by Student's t-test with a significance level of 0.05. A

comparison of the percentage of viruses adsorbed in groundwater and various virus characteristics (i.e. isoelectric point, type of interaction, and structure) was subjected to regression analysis to determine correlations. Regression analysis was also used to determine the effect of soil SCOD retention on virus mobilization in soil columns. All analysis was performed using ProStat© v3.5.

Results

Soil Characteristics

The soil used in the batch and column experiments differed in composition (Table 6-2). Soil used for batch and column studies differed in the amount and type of sand. The amount of silt and clay, the reactive species in soil, was higher in the soil used for batch studies than in the column studies.

Batch Studies

The adsorption of viruses in groundwater, influent and effluent to soil are shown in Figure 6-1. When the viruses were suspended in groundwater, there was greater adsorption of ΦX174 (> 99 %) and poliovirus 1 (96 %) than of MS2 (72 %) and PRD1 (48 %) (Table 6-3). In influent, adsorption decreased to 17 % for MS2 and < 1 % for PRD1. The presence of influent also decreased the adsorption of ΦX174 (78 %), but had little effect on the adsorption of poliovirus 1 (93 %). The adsorption of MS2 (45 %) and PRD1 (8 %) was less in effluent than in groundwater, but greater than in influent. The adsorption of ΦX174 and poliovirus 1 was similar in influent and effluent. These results indicate that the adsorption of viruses was influenced by the presence of effluent and influent and by the type of virus.

The phages adsorbed to soil in the presence of groundwater were desorbed by influent (Table 6-4). The percent adsorption to soil observed for MS2 and PRD1 in

groundwater was lower than for initial adsorption experiments. This difference may be due to variable soil composition during subsampling. The percentage of all phages detached by influent was significantly higher ($P < 0.05$) than the percentage detached by effluent or groundwater. The percent eluted by either effluent or groundwater was not significantly different ($P < 0.05$) for MS2 and PRD1. Poliovirus 1 adsorbed to the soil was not eluted by groundwater, influent or effluent. These results show that, except for poliovirus 1, untreated wastewater can detach more adsorbed phages from soil as compared with anaerobically treated wastewater.

Column Studies

The adsorption of viruses in groundwater, influent and effluent to soil columns is shown in Figure 6-2. The adsorption pattern for the viruses studied was similar to that observed in the batch studies in that the adsorption of ΦX174 (97 %) and poliovirus 1 (99 %) in groundwater was greater than the adsorption of MS2 (88 %) and PRD1 (54 %). In contrast with the batch studies, the difference between adsorption of MS2 and PRD1 in the presence of influent and effluent was small.

As observed in the batch studies, influent was found to mobilize adsorbed viruses (Table 6-5). Mobilization of MS2, PRD1 and ΦX174 by influent was significantly higher ($P < 0.05$) than by effluent or rainwater. Influent mobilized 2 % of poliovirus 1, whereas no mobilization was observed with effluent or rainwater.

Conductivity and pH

The initial pH of the soil samples was considerably lower than the pH of groundwater, rainwater, influent and effluent samples (Table 6-6). In both the batch and column experiments, there was an increase in pH in all samples following mixing of soil with one of the solutions described above. However, we found that normalizing the pH

of the soil to pH 7.0 during the experiment did not affect adsorption and elution (Table 6-7). Furthermore, during percolation, conductivity, or ionic strength, of the soil samples decreased with rainwater and increased with influent and effluent (Table 6-6).

Attachment and Detachment Mechanisms

Soluble organics. MS2 elution displayed a positive correlation with soil SCOD ($r = 0.936$) (Figure 6-3). PRD1 ($r = 0.103$) and ΦX174 ($r = 0.037$) displayed a positive, but low correlation with soil SCOD.

Wastewater fractionation. To determine the size of the compound(s) in influent and effluent affecting virus adsorption to soil, dialysis was used to remove compounds smaller than 100 kDa, 10 kDa, and 1 kDa from the wastewaters. Viruses were inoculated into the wastewater samples that retained compounds larger than the indicated MWCOs.

In samples of influent where molecular weight fractions larger than 10 kDa were retained, adsorption of MS2 (Figure 6-4) was similar to adsorption in groundwater for batch studies (Figure 6-1). This result suggests that compounds in the influent that were smaller than 10 kDa were mainly responsible for interfering with adsorption of MS2 to soil. Adsorption did not significantly increase in the influent where compounds larger than 1 kDa were retained. However, adsorption of MS2 to soil was higher in the effluent where compounds larger than 1 kDa were retained, as compared with adsorption with groundwater in batch studies. These results suggest that in the influent, removal or alteration of compounds between 10 kDa and 1 kDa interferes with adsorption of MS2 to soil.

The adsorption of PRD1 to soil was impacted by different fractions of influent and effluent (Figure 6-5). In the effluent, retention of compounds larger than 10 kDa did not

interfere with adsorption of PRD1 to soil. In the influent, retention of compounds larger than 100 kDa did not interfere with adsorption of PRD1 to soil. These results suggest that compounds in the influent, between 100 kDa and 10 kDa, affect adsorption of PRD1 to soil.

The adsorption pattern of ΦX174 in fractionated wastewater to soil is shown in Figure 6-6. In both the influent and effluent, retention of compounds larger than 100 kDa did not interfere with adsorption of ΦX174 to soil. There were no significant differences observed in adsorption between native wastewater and wastewater retaining compounds larger than 10 kDa and 1 kDa. This finding suggest that compounds in the influent, between 100 kDa and 10 kDa, interefere with adsorption of ΦX174 to soil.

Influent compounds smaller than 100 kDa and 10 kDa were transferred to groundwater, by dialyzing 20 ml of groundwater with 100 kDa and 10 kDa MWCOs against 1 L of influent Viruses were inoculated into groundwater, dialyzed groundwater, influent, and influent used for dialysis. The samples were mixed with soil as previously described for batch studies. Adsorption patterns for PRD1 and ΦX174 are shown in Figure 6-7. Adsorption of PRD1 and ΦX174 to soil decreased when suspended in groundwater containing influent compounds smaller than 100 kDa. Adsorption of PRD1 in influent and groundwater dialyzed with influent to soil was similar. Adsorption of ΦX174 to was lower using suspensions of groundwater dialyzed with influent than in raw influent. Furthermore, a slight increase in adsorption was observed for both viruses suspended in influent used for dialysis. These results, along with the results from Figures 6-5 and 6-6, demonstrate the interference of influent compounds smaller than 100 kDa on adsorption of PRD1 and ΦX174 to soil.

The influence of influent compounds smaller than 10 kDa on adsorption of MS2 to soil was also performed. MS2, suspended in groundwater containing influent compounds smaller than 10 kDa, was not detected in the supernatants after mixing with the soil for 1 h. Furthermore, MS2 was not detected in the initial stock solutions of groundwater containing influent compounds smaller than 10 kDa. To determine if influent compounds smaller than 10 kDa inactivated MS2, groundwater and groundwater dialyzed against influent was inoculated with MS2. A 300 µl aliquot was taken at 10 min intervals for 1 h. Influent compounds smaller than 10 kDa were found to inactivate MS2 (Figure 6-8). Inactivation was not observed in experiments using the whole fraction of influent. This may be due to interaction between particulate organics and the compounds causing inactivation, thereby preventing inactivation of MS2.

Influence of isoelectric point on virus adsorption to soil. Adsorption of viruses in groundwater to soil in batch studies correlated with isoelectric point ($r = 0.973$) (Figure 6-9).

Influence of pH on virus adsorption to soil. To determine the effect wastewater had on virus adsorption at pH values below the pI, the pH of the groundwater and wastewaters were adjusted to 3.5. Adsorption to soil by all three viruses in groundwater and wastewater adjusted to pH 3.5 increased to > 99 % (Table 6-8).

Hydrophobic and electrostatic interactions. Viruses utilize hydrophobic and electrostatic interactions to bind to soil. To determine the type of interactions used by the viruses under study, ionic and nonionic detergents were used. Ionic and nonionic detergents were added to groundwater and wastewater to a final concentration of 0.01 %, the maximum concentration where viruses remained active. The detergent solutions were

inoculated with viruses and batch adsorption studies were performed as previously described.

All three detergents increased the adsorption of MS2 and PRD1 suspended in groundwater to soil (Figure 6-10). However, adsorption of ΦX174 in groundwater decreased in the presence of Tween 80, was unaffected by SDS, and increased to greater than 99% in the presence of HTAB.

The same pattern of adsorption to soil was seen when detergents and viruses were added to influent (Figure 6-11). Again, all three detergents increased the adsorption of MS2 and PRD1 to soil. The detergents had little to no effect on adsorption of ΦX174 to soil.

The three detergents in effluent had little effect on the adsorption of MS2 and ΦX174 to soil (Figure 6-12). All three detergents in effluent increased the adsorption of PRD1 to soil.

The effect of ionic and nonionic detergents on virus detachment from soil was determined in batch studies first adsorbing viruses in groundwater to soil. Next, the soil samples, with adsorbed viruses, were mixed with groundwater and groundwater with 0.01% of each detergent. The detergents eluted less than 40% of the adsorbed viruses in all tests. Detachment of MS2 and ΦX174 from soil by Tween 80 and SDS was not significantly different ($P < 0.05$) (Table 6-9). Detachment of PRD1 by Tween 80 was significantly higher ($P < 0.05$) than the ionic detergents. Mixing the soil with HTAB in groundwater did not result in detachment of any viruses.

Remaining attached viruses were recovered by mixing samples with 10 ml 3% BE for 30 min. The percentage of viruses recovered during the secondary elution are shown

in Table 6-10. Recovery of MS2 and PRD1 with 3% BE from soil batches receiving Tween 80 as the primary eluent was significantly higher ($P < 0.05$) than batches receiving SDS and HTAB as the primary eluent. There was no significant difference ($P < 0.05$) between the recoveries of ΦX174 from soil batches receiving Tween 80 and SDS as the primary eluents. Recovery of all viruses was lowest from batches receiving HTAB as the primary eluent.

To determine if the detergents influenced virus adsorption by interacting with soil binding sites, soil samples were pretreated with groundwater or groundwater with 0.01 % detergent. Viruses in groundwater were mixed with pretreated soil samples in batch studies. Adsorption of MS2 to soil pretreated with Tween 80 and SDS decreased (Figure 6-13). Adsorption of PRD1 and ΦX174 to soil pretreated with Tween 80 decreased, but was unaffected in soil pretreated with SDS. Adsorption by all viruses to soil pretreated with HTAB was greater than 90 %.

The effect of each pretreatment was determined by calculating the difference in adsorption between unaltered soil and pretreated soil. Pretreatment with Tween 80 caused a significant decrease ($P < 0.05$) in adsorption of MS2 and PRD1 to soil (Table 6-10). Adsorption of ΦX174 to soil pretreated with Tween 80 and SDS was not significantly different ($P < 0.05$). Pretreatment with HTAB increased adsorption of all viruses > 95 %.

Recovery of viruses from soil pretreated with detergents is shown in Table 6-10. Recovery of MS2 and PRD1 was significantly higher ($P < 0.05$) from soil pretreated with Tween 80 than from soil pretreated with SDS and HTAB. Recovery of MS2 and PRD1

was lowest from soil pretreated with HTAB. Recoveries of ΦX174 from soil were not significantly different ($P < 0.05$) between detergent pretreatments.

Risk Assessment of Viruses Following Land Application

Infectious dose data for adenovirus (PHAC, 1999), rotavirus (Graham et al., 1987), and Norwalk virus (Schaub and Oshiro, 2000) was used to determine the probability of infection following exposure to groundwater where influent and effluent are applied to land. Viruses present in influent applied to land had an 85% probability of causing an infection following exposure to groundwater contaminated with influent (Table 6-11). However, following digestion, the probability of infection dropped to 12%. Furthermore, there was an 88% probability of acquiring an infection from groundwater where influent was land applied resulting in mobilization of viruses attached to soil; whereas application of digested wastewater reduced the probability to 5%.

Survival of Viruses in Soil

Survival of viruses suspended in groundwater, influent, and effluent applied to soil is shown in Figure 6-14. MS2, PRD1, and ΦX174 decreased during week 1 in all solutions and were not detected thereafter. Poliovirus 1 LSc showed the highest decrease in effluent during week 1, as compared with groundwater and influent. After week 2, decline of poliovirus 1 LSc was similar for all three solutions. Poliovirus 1 LSc was not detected after week 4.

Discussion and Conclusions

The adsorption of viruses to soil and virus transport through soil columns has been reported in several studies (Chu et al., 2003, McLeod et al., 2001, Quanrud et al., 2003, Zhuang and Jin, 2003). Factors that influence virus adsorption include the characteristics

of the viruses, the properties of the soil, and the chemical composition of the solution that contains the viruses.

In the batch studies, we have confirmed that the isoelectric point of viruses influences their adsorption to soil ($r = 0.973$). Also, the presence of untreated wastewater intereferes with virus adsorption to soil. Further more untreated wastewater promotes mobilization of viruses adsorbed to the soil columns. For MS2, mobilization or detachment from soil is influenced by the amount of soluble organics. This result is consistent with characteristics of Group I viruses, where adsorption is influenced by pH and the presence of organics (Gerba and Goyal, 1981). However, the correlation with soluble organics retained by the soil and mobilization of ΦX174, another Group I virus, was low suggesting that other factors in the soil or wastewater interferes with adsorption.

The viruses studied demonstrated varying adsorption and elution behavior. Changes in pH of suspending solution, soil columns, and natural soils have been shown to influence the adsorption and elution behavior of viruses (Goyal and Gerba, 1979, Kinoshita et al., 1993). In the current study, increases in pH of the inoculated wastewaters were observed during the experiment. However, the observed pH increases did not influence virus adsorption and elution behavior. Additionally, when the pH of the suspending solutions was adjusted below the pI of all the viruses tested, adsorption for all viruses to soil was greater than 99%.

The presence of dissolved ions in the soil may also influence adsorption and elution of viruses (Hurst et al., 1980). In this study, the conductivity of the soil decreased when mixed with rainwater and increased when mixed with influent and effluent. Increased mobilization of phages was observed with influent as compared with effluent. However,

the conductivity of the soil mixed with effluent was slightly higher than influent. Furthermore, removal of salts from the wastewater during dialysis experiments using 1 kDa MWCO dialysis tubing did not increase adsorption. Therefore, changes in ionic strength did not appear to be the predominating factor contributing to the mobilization patterns of phages observed in the current study.

Compared with untreated wastewater, certain fractions of treated and untreated wastewater resulted in increases in virus adsorption to soil. Retention of compounds larger than 10 kDa in the influent yielded the most dramatic increase in adsorption of MS2 to soil, as compared to unaltered influent. Transferring influent compounds smaller than 10 kDa into groundwater caused inactivation of MS2. The results from the current study show that influent compounds smaller than 10 kDa inactivates MS2. Inactivation was not observed in unaltered influent samples. This may be due to interactions with organics by the virus or the inhibitory compounds, interfering with the inhibitory affect.

Retention of compounds larger than 100 kDa in the influent resulted in increased adsorption of PRD1 and ΦX174 to soil, as compared with unaltered influent. Transferring influent compounds smaller than 100 kDa into groundwater caused decreased adsorption to soil for both viruses, as compared to unaltered groundwater. The adsorption of PRD1 to soil was similar when viruses were suspended in influent and in groundwater containing influent compounds smaller than 100 kDa. Adsorption of both viruses to soil increased slightly when suspended in influent where compounds smaller than 100 kDa were lost to groundwater. The current study shows that influent compounds smaller than 100 kDa interfere with adsorption of PRD1 and ΦX174 to soil.

Ionic and nonionic detergents were used to determine the influence of hydrophobic and electrostatic interactions for adsorption to soil by the viruses under study. Groundwater and wastewater with a final detergent concentration of 0.01 % impacted the adsorption of added viruses to soil. Tween 80 in groundwater resulted in the greatest decrease in adsorption of ΦX174 to soil as compared with Tween 80 in influent and effluent. However, Tween 80 increased adsorption of MS2 and PRD1. As demonstrated by retention of soluble COD by soil, the soil can react with soluble organics. Interaction between Tween 80 (nonionic detergent) and the soil is also possible, which would result in decreased virus adsorption to soil. However in the current study, adsorption to soil increased for MS2 and PRD1 in wastewater containing detergents. Alternatively, pretreatment of soil with Tween 80 caused a decrease in adsorption of MS2 and PRD1. Furthermore, Tween 80 was able elute MS2 and PRD1 attached to soil. Adsorption sites may have become unavailable during pretreatment with detergents. Also, hydrophobic and electrostatic interactions between soil and attached viruses may have been interrupted upon introducing Tween 80 to the medium. The effect of soil pretreatment and elution with Tween 80 demonstrates the variable interactions utilized by MS2 and PRD1. Soluble organics in untreated wastewater may bind to available sites on soil. As sites become unavailable for binding, adsorption to soil may be achieved through hydrophobic or electrostatic interactions. During anaerobic digestion, the concentration of soluble organics is decreased, which may lead to a decrease in the interference of adsorption by hydrophobic interactions. Furthermore, for PRD1, hydrophobic and some weak electrostatic interactions with the soil may be disrupted by the presence of a specific

soluble component(s) of the influent smaller than 100 kDa, leaving the virus to utilize weak hydrophobic or electrostatic interactions for adsorption to soil.

The use of anionic and cationic detergents in groundwater and wastewater, inoculated with viruses, increased virus adsorption to soil. The use of SDS (anionic) in groundwater and wastewater increased the adsorption of MS2 and PRD1 to soil. The viruses and SDS have a net negative charge, which may have resulted in charge repulsion from the detergent in solution. Conversely, pretreating and eluting with SDS caused decreased adsorption and detachment, respectively. The effect of SDS was more pronounced for PRD1 in both cases than for MS2. This result further suggests that these viruses may use either hydrophobic or electrostatic interactions to bind to soil. The use of HTAB (cationic) increased adsorption and was unable to elute any of the viruses used in the study. The presence of cations is known to increase virus adsorption to soil (Goyal and Gerba, 1979, Hurst et al., 1980). Our results show that in the presence of HTAB, viruses increased adsorption to soil by greater than 95 % and that HTAB did not cause detachment of adsorbed viruses.

The survivability of viruses in groundwater, untreated, and treated wastewater was demonstrated in the current study. The phages only remained active for 1 week following inoculation, whereas poliovirus 1 LSc persisted for up to 4 weeks. This result suggests that some retained viruses may persist in the soil for prolonged periods. However the current study demonstrated how anaerobic treatment of flushed dairy manure wastewater causes less interference and less desorption of viruses. Therefore, during prolonged periods, active viruses may be retained in the soil following application of treated wastewater.

The current study provided evidence demonstrating the adsorption and desorption behavior of selected viruses. In both the batch and column studies, untreated wastewater was shown to decrease viral adsorption to sandy soil. These results suggest that viruses present in untreated dairy manure wastewater may have potential for groundwater contamination during land application. Furthermore, untreated wastewater was shown to increase the release of viruses attached to soil. Thus, land application of untreated dairy manure wastewater may increase the movement of retained viruses through soil, potentially contaminating groundwater.

The present study demonstrates the benefit of anaerobic treatment in regards to reducing potential groundwater contamination by viruses in sandy soils. Anaerobic treatment alters the physicochemical characteristics of flushed dairy manure wastewater (Wilkie et al., 2004). The use of untreated wastewater may cause migration of viruses through the soil matrix by interfering with adsorption mechanisms. As demonstrated with MS2, untreated wastewater may disrupt interactions between the soil and MS2 due to the increased amount of soluble organics contributed by untreated wastewater to the soil. Furthermore, influent compounds smaller than 10 kDa was shown to cause inactivation of MS2. Untreated wastewater also interfered with adsorption of PRD1 and ΦX174. This interference is accomplished by component(s) smaller than 100 kDa in the wastewater. Anaerobic digestion lowers the soluble organic content and may also remove the fraction interfering with adsorption, resulting in increased virus retention and decreased virus mobilization in the soil. We conclude from the current study that anaerobic treatment of flushed dairy manure wastewater increases the retention of some

viruses to soil and decreases the transport of some adsorbed viruses through the soil matrix, thereby decreasing the potential for groundwater contamination.

Table 6-1 Characteristics of viruses used in the study.

Virus	Family	pI[a]	Diameter, nm	Structure	Nucleic Acid	Lipid content, %	Interaction[b]	Group[c]
MS2	Leviviridae	3.9	24	icosahedral	s/s (+) strand RNA	0	Strong hydrophobic/ weak electrostatic	I
PRD-1	Tectiviridae	4.2	63	complex	linear d/s DNA	16	Hydrophobic/ Electrostatic	ND*
ΦX174	Microviridae	6.6	25 - 27	icosahedral	circular s/s (+) DNA	0	weak hydrophobic & electrostatic	I
Poliovirus 1 LSc	Picornaviridae	4.5 & 7.5	30	icosahedral	s/s (+) strand RNA	0	electrostatic	II

*ND, Not determined

[a] Dowd, S. E. *et al.* (1998).

[b] Shields, P.A and S. R. Farrah. (2002).

[c] Gerba C. P. and S. M. Goyal. (1981).

Table 6-2 Characteristics of test soil.

Parameter	Whole soil	Test soil for experiment	
		Batch	Column
Sand, 2 - 1 mm (%)	0.1	0.0	0.0
Sand, 1 - 0.5 mm (%)	5.2	0.0	6.3
Sand, 0.5 - 0.25 mm (%)	41.9	0.1	52.9
Sand, 0.25 - 0.1 mm (%)	41.7	40.4	35.0
Sand, 0.1 - 0.05 mm (%)	5.4	40.0	1.2
Sand, total (%)	94.3	80.5	95.4
Silt, (%)	2.8	15.2	3.2
Clay, (%)	2.9	4.3	1.4

Table 6-3 Adsorption of Viruses in groundwater and wastewater to soil.

Virus	Adsorption to soil, %		
	Groundwater	Influent	Effluent
MS2	72 ± 3^{A}	17 ± 10^{C}	45 ± 12^{B}
PRD1	48 ± 5^{A}	2 ± 0^{B}	8 ± 8^{B}
ΦX174	100 ± 0^{A}	78 ± 14^{B}	$81 \pm 16^{A,B}$
Poliovirus 1	96 ± 0^{A}	93 ± 1^{A}	93 ± 2^{A}

Values are average ± standard deviation (n=6).

Notes: Solutions were groundwater; influent (flushed dairy manure wastewater) and effluent (anaerobically digested flushed dairy manure wastewater).

[A-B]Values with the same letter within the same row are not significantly different ($P < 0.05$).

Table 6-4 Elution of viruses adsorbed to soil.

Virus	Initial amount, PFU	Adsorbed, %	Eluted, % of adsorbed			
			GW	Influent	Effluent	3% BE
MS2	$3.2 \pm 0.4 \times 10^4$	44 ± 25	41 ± 26^{B}	100 ± 0^{A}	50 ± 12^{B}	89 ± 23^{A}
PRD1	$1.1 \pm 0.1 \times 10^6$	33 ± 16	33 ± 12^{B}	97 ± 4^{A}	37 ± 10^{B}	100 ± 0^{A}
ΦX174	$1.1 \pm 0.1 \times 10^4$	99 ± 0.2	8 ± 2^{D}	92 ± 2^{A}	21 ± 5^{C}	47 ± 9^{B}
Poliovirus 1 LSc	$5.1 \pm 1.9 \times 10^4$	99 ± 0.5	0	0	0	37 ± 1

Notes: Viruses in 2ml of groundwater (GW) were mixed with 1 g of soil for 1 hr. The samples were centrifuged and the supernatant fraction was assayed for viruses. The soil pellets were mixed with 10 ml groundwater, influent, effluent, or 3 % beef extract (BE) for 30 min. The samples were centrifuged and the supernatant fraction assayed to determine the percentage of virus elution.

Values are average ± standard deviation (n=6).

[A-C]Values with the same letter within the same row are not significantly different ($P < 0.05$).

Table 6-5 Elution of viruses adsorbed in soil columns.

Virus	Initial amount, PFU	Adsorbed, %	Eluted, % of adsorbed			
			Rainwater	Influent	Effluent	10% BE
MS2	$7.6 \pm 1.6 \times 10^3$	91 ± 1	1 ± 0.6^{C}	63 ± 13^{A}	16 ± 3^{B}	61 ± 10^{A}
PRD1	$2.4 \pm 0.3 \times 10^5$	50 ± 6	21 ± 7^{C}	79 ± 14^{A}	36 ± 7^{B}	40 ± 8^{B}
ΦX174	$1.4 \pm 0.2 \times 10^5$	98 ± 0.3	10 ± 5^{B}	48 ± 20^{A}	5 ± 4^{B}	43 ± 7^{A}
Poliovirus 1 LSc	$1.8 \pm 0.1 \times 10^4$	99 ± 0.1	0	2 ± 0^{B}	0	41 ± 9^{A}

Notes: Viruses were inoculated into 1 pore volume (20 ml) of groundwater and added to soil columns. The percolate was collected and assayed to determine the percentage of virus adsorption. Six pore volumes of rainwater was added to each column. The percolates were collected and assayed for viruses. Each column then received six pore volumes of either rainwater, influent, or effluent. The percolates were collected and assayed to determine the percentage of viruses eluted. An additional column received 1 pore volume of 10% beef extract (BE) to serve as a control.

Values are average ± standard deviation (n=3).

$^{A-C}$Values with the same letter within the same row are not significantly different ($P < 0.05$).

Table 6-6 Conductivity and pH changes in soil during batch and column experiments.

Sample	pH		Conductivity, μS/cm
	Batch	Column	Column
Soil	5.02	5.02	473
Groundwater	7.71	7.71	353
Groundwater + Soil	6.86	6.50	452
Rainwater	NA	7.12	39
Rainwater + Soil	NA	5.81	122
Influent	7.77	7.33	3370
Influent + Soil	7.27	8.04	628
Effluent	7.20	7.49	3600
Effluent + Soil	7.41	7.82	700

Notes: Influent, flushed dairy manure wastewater; Effluent, anaerobically digested flushed dairy manure wastewater; NA, not applicable.

Table 6-7. Adsorption of viruses in groundwater and wastewater to soil adjusted to pH 7.0.

Virus	Initial amount, PFU	Solution					
		Groundwater		Influent		Effluent	
		Adsorbed, %	Eluted, % of Initial	Adsorbed, %	Eluted, % of Initial	Adsorbed, %	Eluted, % of Initial
MS2	$4.1 \pm 0.6 \times 10^6$	63 ± 8	36 ± 10	17 ± 8	10 ± 3	39 ± 8	25 ± 7
PRD1	$1.7 \pm 0.6 \times 10^5$	35 ± 6	16 ± 8	3 ± 0	3 ± 0	9 ± 1	4 ± 2
ΦX174	$1.8 \pm 0.5 \times 10^3$	91 ± 2	74 ± 10	78 ± 14	62 ± 10	80 ± 12	69 ± 11
Poliovirus 1 LSc	$5.7 \pm 0.4 \times 10^3$	98 ± 0.4	75 ± 9	89 ± 12	73 ± 11	93 ± 12	74 ± 13

Notes: Batch adsorption studies and elution with 3% beef extract were performed as described in the text. The pH was maintained at 7.0 during the experiment.

Values are average ± standard deviation (n=3)

Table 6-8 Influence of pH on adsorption of viruses to soil.

Sample	pH	Adsorption, %		
		MS2	PRD1	ΦX174
Groundwater	7.68	82.9 ± 6.6	56.6 ± 4.4	99 ± 0.7
	3.50	99.8 ± 0	100 ± 0	99.2 ± 0.9
Influent	7.75	27.7 ± 3.2	6.7 ± 2.4	76.5 ± 1.9
	3.50	99.9 ± 0	100 ± 0	99.9 ± 0
Effluent	7.24	42.9 ± 2.8	16.3 ± 3.4	84.8 ± 1.6
	3.50	99.7 ± 0.1	100 ± 0	99.9 ± 0

Notes: Groundwater, influent, and effluent were inoculated with viruses. Groundwater and wastewater adjusted to pH 3.5 with 1 N HCl were inoculated with viruses. Samples were mixed with soil as described for batch studes. Adsorption values are average ± standard deviation (n = 3).

Table 6-9 Elution of viruses adsorbed to soil by detergents in groundwater.

Virus	Adsorbed, % of Initial	Eluted, % of Adsorbed					
		0.01% Tween 80		0.01% SDS		0.01% HTAB	
		Detergent	3% BE	Detergent	3% BE	Detergent	3% BE
MS2	89 ± 4	12 ± 2 ^{A}	53 ± 15^{a}	8 ± 1 ^{A}	38 ± 12^{b}	0	10 ± 3^{c}
PRD1	46 ± 2	30 ± 6 ^{A}	40 ± 6^{a}	18 ± 3 ^{B}	25 ± 8^{b}	0	8 ± 2^{c}
ΦX174	86 ± 4	10 ± 4 ^{A}	20 ± 5^{b}	7 ± 2 ^{A}	38 ± 5^{a}	0	4 ± 1^{c}

Notes: Viruses in groundwater were absorbed to 1 g soil samples. A cationic (hexadecyltrimethylammonium bromide, HTAB), anionic (sodium dodecyl sulfate, SDS), and nonionic (polyoxyethylene sorbitan monooleat, Tween 80) detergent was added to groundwater to a final concentration of 0.01 %. The detergent solutions were mixed with the soil samples and the percentage of viruses recovered determined.

$^{A-B}$ Values with the same letter within the same row are not significantly different ($P < 0.05$).

Values are averages ± standard deviation (n = 3)

Table 6-10 Recovery of viruses with 3% BE from soil pretreated with detergents.

Virus	Initial amount, PFU	Soil Pretreatment					
		0.01% Tween 80		0.01% SDS		0.01% HTAB	
		Adsorbed, %	Eluted, % of Adsorbed	Adsorbed, %	Eluted, % of Adsorbed	Adsorbed, %	Eluted, % of Adsorbed
MS2	$9.9 \pm 1.9 \times 10^4$	53 ± 7	97 ± 20^A	66 ± 9	76 ± 42^B	95 ± 1	52 ± 12^c
PRD1	$3.8 \pm 0.5 \times 10^5$	20 ± 6	91 ± 8^A	39 ± 11	52 ± 22^B	96 ± 1	36 ± 8^C
ΦX174	$8.3 \pm 1.5 \times 10^5$	77 ± 3	34 ± 7^A	81 ± 7	30 ± 18^A	96 ± 1	34 ± 9^A

Notes: Soil samples (1 g) were pretreated with detergents and mixed with viruses as previously described. Attached viruses were recovered by mixing soil samples with 3% BE for 30 min.

$^{A-C}$ Values with the same letter within the same row are not significantly different ($P < 0.05$).

Values are averages $\pm$ standard deviation (n = 3).

Table 6-11 Estimated risk of infection from exposure to groundwater following land application of wastewater.

	Probability of infection, %	
	Virus introduced by wastewater	Virus mobilized by wastewater
Influent	85	88
Effluent	12	5

Notes: Infectious dose data for adenovirus (PHAC, 1999), rotavirus (Graham *et al* 1987), and Norwalk virus (Schaub and Oshiro, 2000) was used calculating probability of infection. Probability of infection are based on adsorption characteristics of MS2 in batch studies.

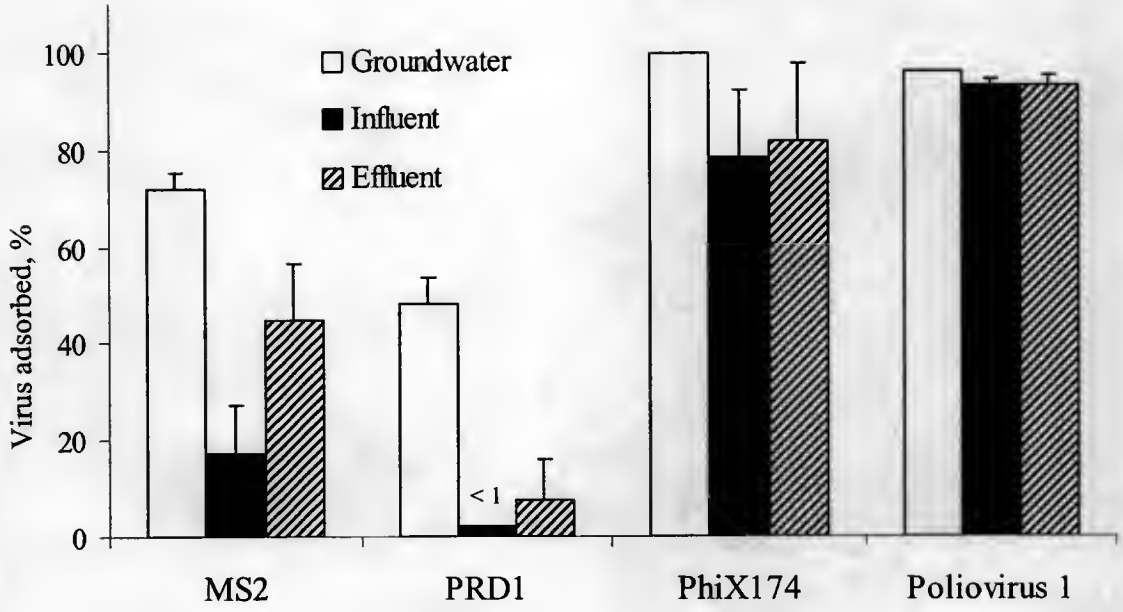

Figure 6-1 Adsorption of viruses in groundwater and wastewater to soil.

Notes: Two ml of groundwater, flushed dairy manure wastewater (influent) and anaerobically digested manure wastewater (effluent) samples were seeded with virus and mixed with 1 g of soil for 1 hr. The samples were centrifuged and the supernatant fraction was assayed to determine the percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

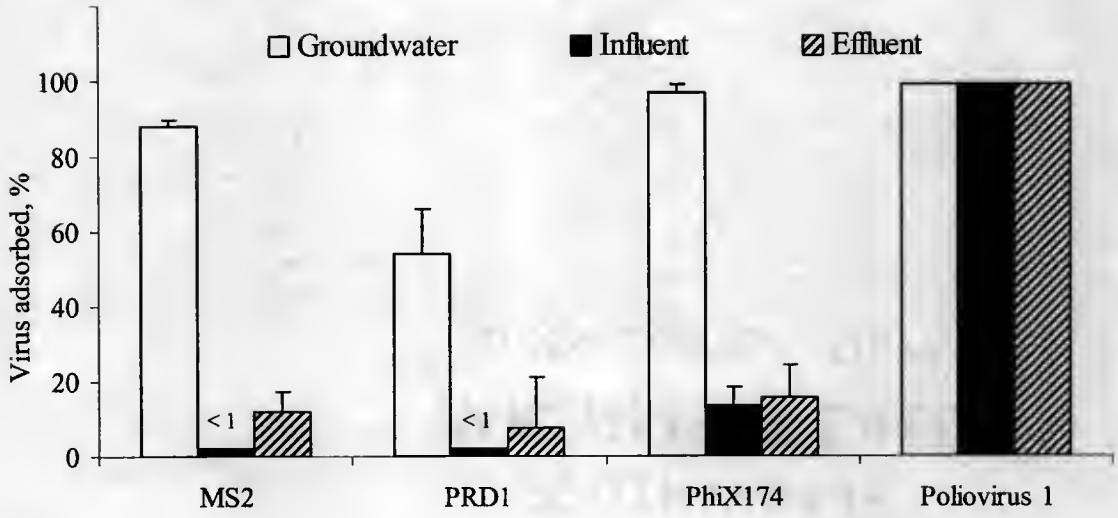

Figure 6-2 Adsorption of viruses in groundwater and wastewater to soil columns.

Notes: Viruses inoculated into one pore volume (20 ml) of groundwater, flushed dairy manure wastewater (influent), and anaerobically digested flushed dairy manure wastewater (effluent) was added to soil columns. The percolates were collected and assayed to determine the percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

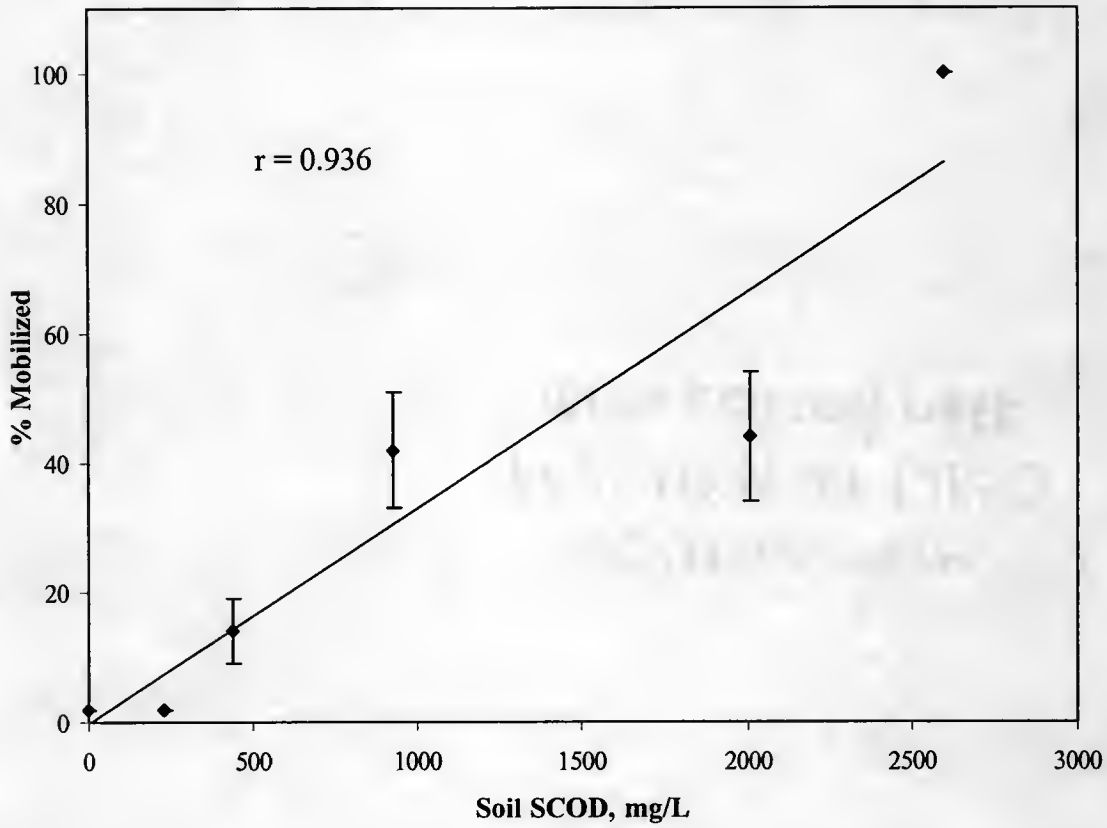

Figure 6-3 Correlation between soluble chemical oxygen demand retained by the soil and MS2 detachment from soil columns.

Notes: Soluble chemical oxygen demand (SCOD) retained by the soil after exposure to wastewater was calculated by subtracting initial wastewater SCOD from SCOD of the wastewater after percolating through the soil column. Soil SCOD was determined for each pore volume where viruses were detected in the percolate. Error bars represent the standard deviation (n = 3) of the percentage of MS2 mobilized through the soil column.

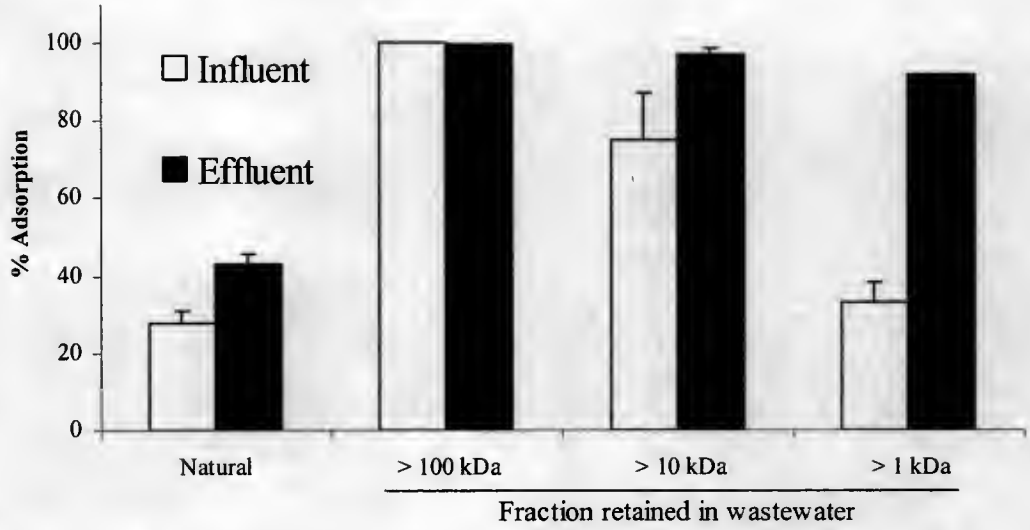

Figure 6-4 Adsorption of MS2 in fractionated wastewater to soil.

Notes: Samples of flushed dairy manure wastewater (influent) or anaerobically digested flushed dairy manure wastewater (effluent) were placed in dialysis tubing with molecular weight cutoffs of 100 kDa, 10 kDa, and 1 kDa. The samples were dialyzed against denionzed water for 3 days at 4°C. Two ml of undialyzed (natural) or dialyzed samples were seeded with virus and mixed with 1 g of soil for 1 hr. The samples were centrifuged and the supernatant fraction was assayed to determine percent adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

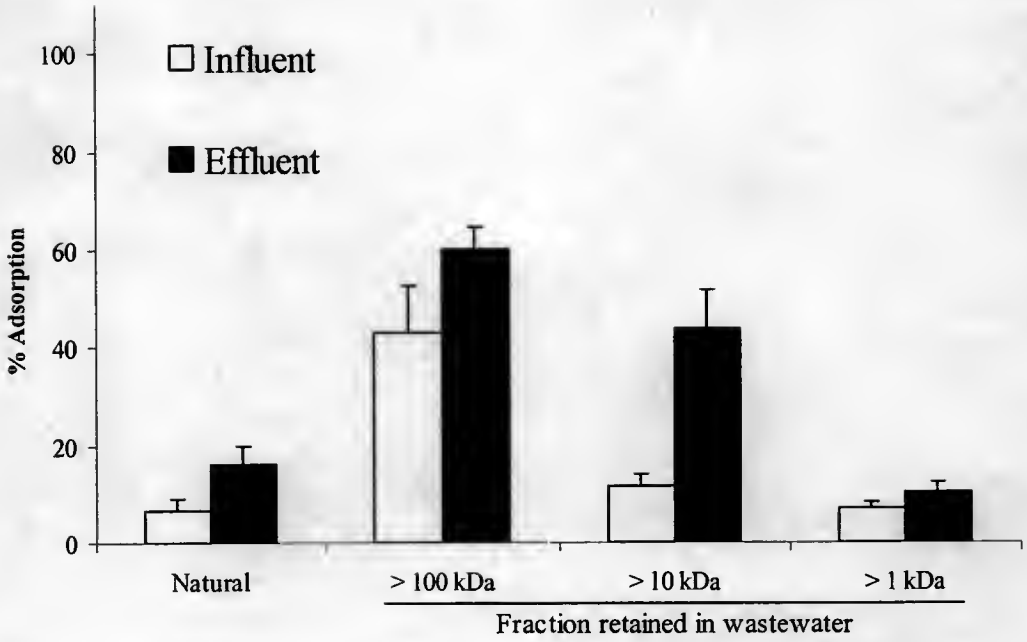

Figure 6-5 Adsorption of PRD-1 in fractionated wastewater to soil.

Notes: Samples of flushed dairy manure wastewater (influent) or anaerobically flushed dairy manure wastewater (effluent) were placed in dialysis tubing with molecular weight cutoffs of 100 kDa, 10 kDa, and 1 kDa. The samples were dialyzed against denionzed water for 3 days at 4°C. Two ml of undialyzed (natural) or dialyzed samples were seeded with virus and mixed with 1 g of soil for 1 hr. The samples were centrifuged and the supernatant fraction was assayed to determine percent adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

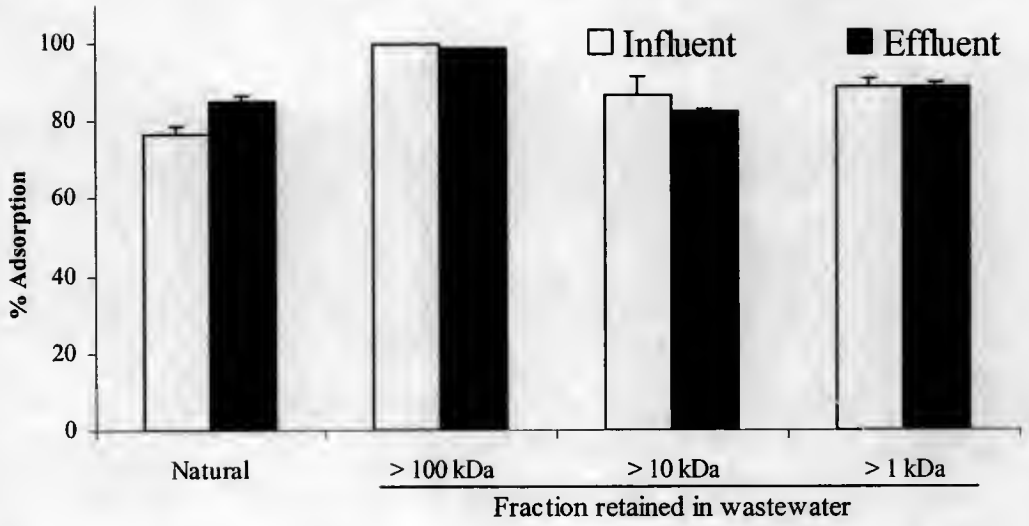

Figure 6-6 Adsorption of ΦX174 in fractionated wastewater to soil.

Notes: Samples of flushed dairy manure wastewater (influent) or anaerobically digested flushed dairy manure wastewater (effluent) were placed in dialysis tubing with molecular weight cutoffs of 100 kDa, 10 kDa, and 1 kDa. The samples were dialyzed against denionzed water for 3 days at 4°C. Two ml of undialyzed (natural) or dialyzed samples were seeded with virus and mixed with 1 g of soil for 1 hr. The samples were centrifuged and the supernatant fraction was assayed to determine percent adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

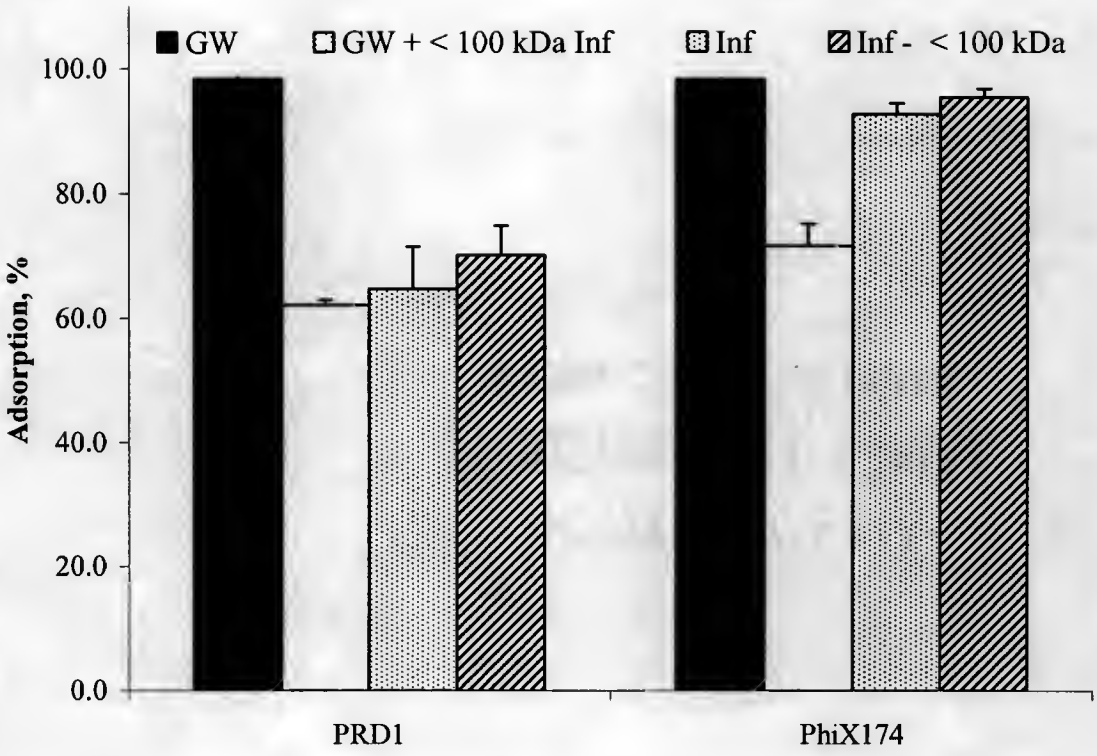

Figure 6-7 Influence of influent compounds smaller than 100 kDa on adsorption of viruses to soil.

Notes: Twenty milliliters of groundwater was placed in 100 kDa MWCO dialysis tubing and dialyzed against 1 L of flushed dairy manure wastewater (influent) for 3 days at 4°C. Two milliliters of groundwater (GW), influent (Inf), dialyzed GW (GW + < 100 kDa Inf), and influent used for dialysis (Inf - <100 kDa) were inoculated with viruses and mixed with 1 g of soil for 1 hr. Samples were centrifuged and the supernatant fraction was assayed to determine percent adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

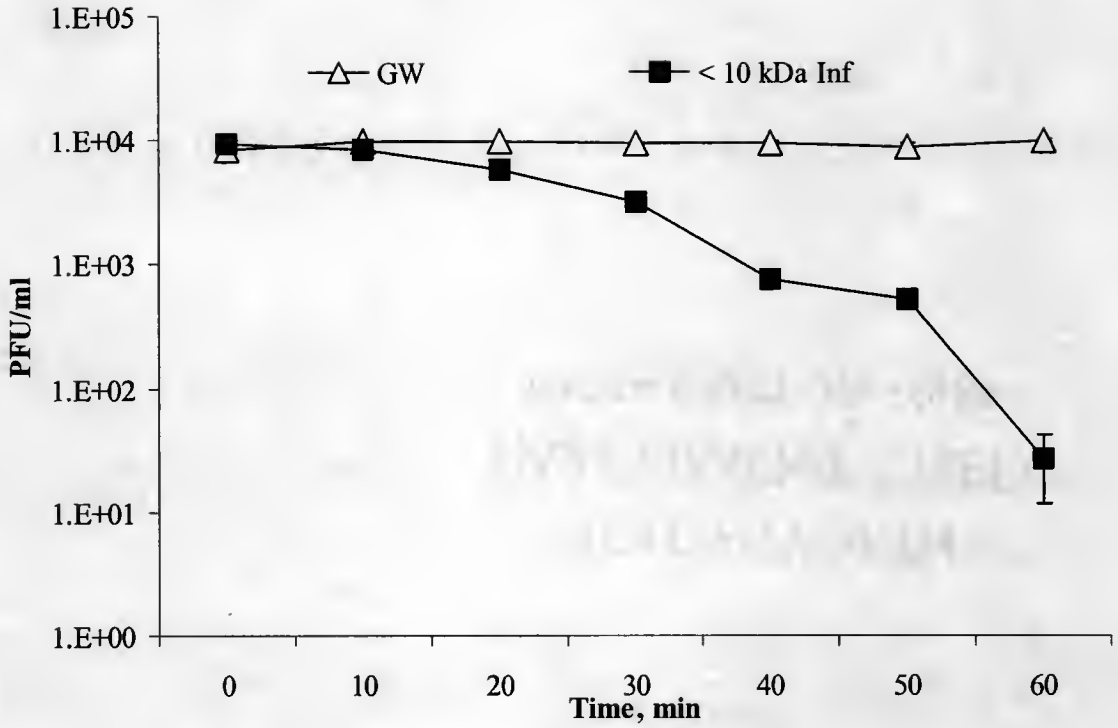

Figure 6-8 Inactivation of MS2 by wastewater compounds smaller than 10 kDa.

Notes: Groundwater (Δ) in 10 kDa MWCO dialysis tubing was dialyzed against 1 L of flushed dairy manure wastewater (influent) for 3 days at 4°C. MS2 was inoculated into groundwater and dialyzed groundwater (■). Virus solutions were assayed at 10 min intervals. Each point is an average and error bars represent standard deviation (n = 3).

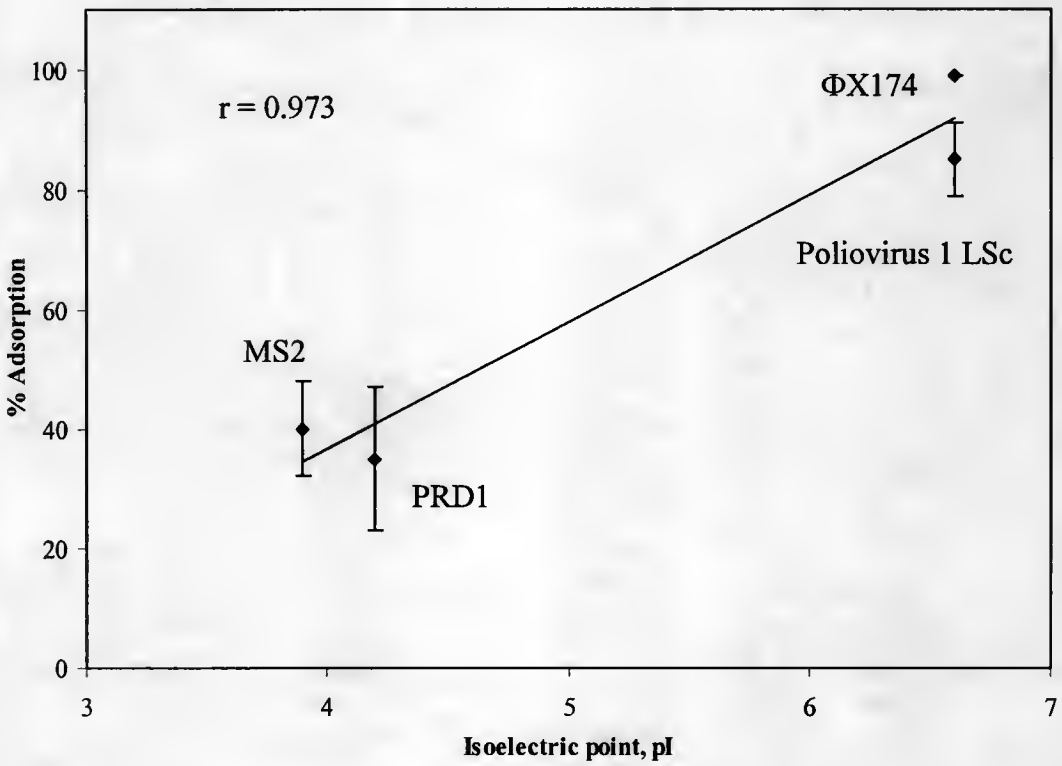

Figure 6-9 Correlation between virus pI and virus adsorption to soil.

Notes: The isoelectric point of MS2, PRD-1, ΦX174, and poliovirus 1 (LSc) was plotted against adsorption of each virus in groundwater to soil. Error bars represent the standard deviation (n = 3) of percent adsorption of each virus in groundwater to soil.

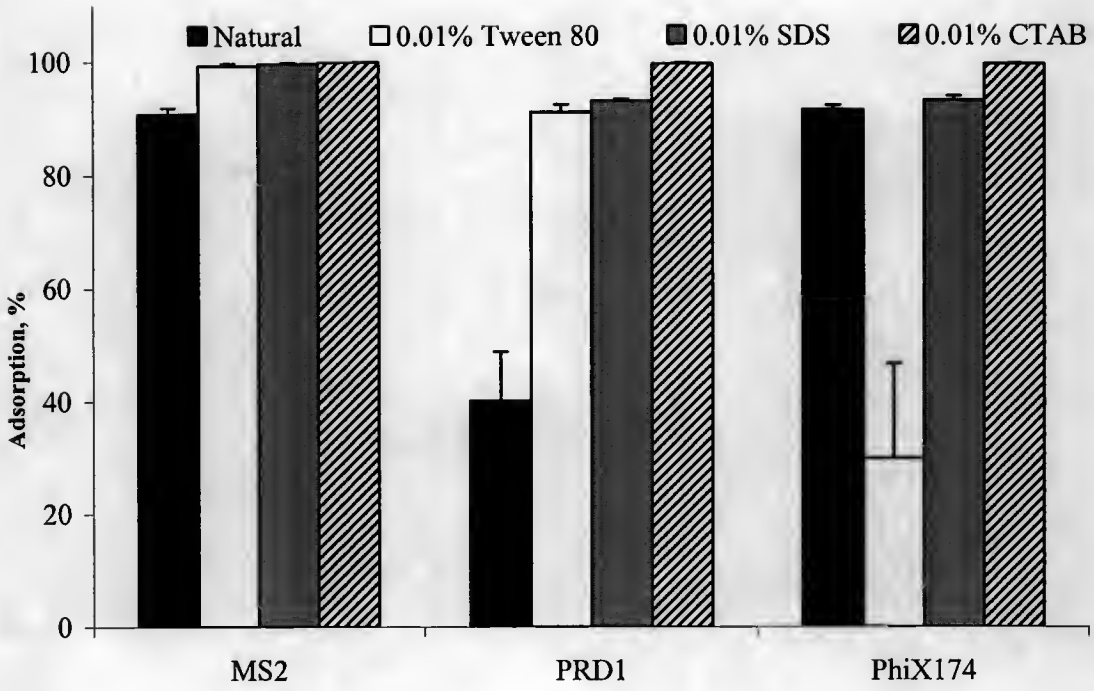

Figure 6-10 Adsorption of viruses in groundwater with 0.01% final concentration of detergents to soil.

Notes: Two milliliters of cationic (hexadecyltrimethylammonium bromide, HTAB), anionic (sodium dodecyl sulfate, SDS), and nonionic (polyoxyethylene sorbitan monooleat, Tween 80) detergent solutions were seeded with viruses and mixed with 1 g of soil for 1 hr. Samples were centrifuged and the supernatant fraction was assayed to determine percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

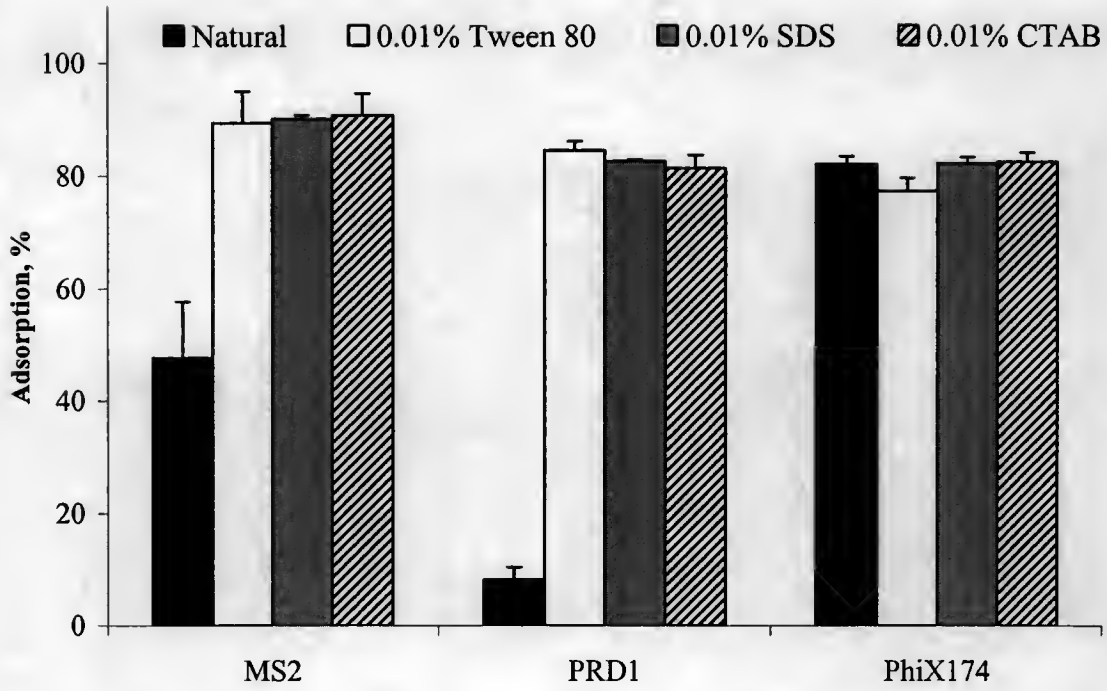

Figure 6-11 Adsorption of viruses in influent with 0.01% final concentration of detergents to soil.

Notes: Two milliliters of cationic (hexadecyltrimethylammonium bromide, HTAB), anionic (sodium dodecyl sulfate, SDS), and nonionic (polyoxyethylene sorbitan monooleat, Tween 80) detergent solutions were seeded with viruses and mixed with 1 g of soil for 1 hr. Samples were centrifuged and the supernatant fraction was assayed to determine percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

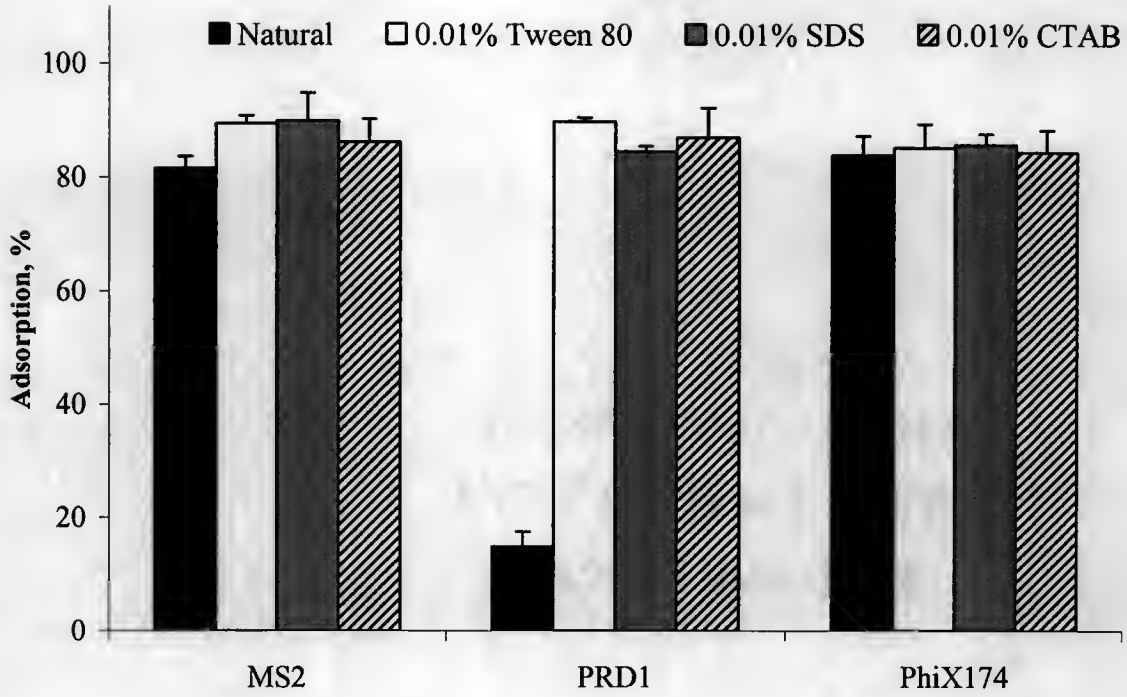

Figure 6-12 Adsorption of viruses in effluent with 0.01% final concentration of detergents to soil.

Notes: Two milliliters of cationic (hexadecyltrimethylammonium bromide, HTAB), anionic (sodium dodecyl sulfate, SDS), and nonionic (polyoxyethylene sorbitan monooleat, Tween 80) detergent solutions were seeded with viruses and mixed with 1 g of soil for 1 hr. Samples were centrifuged and the supernatant fraction was assayed to determine percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

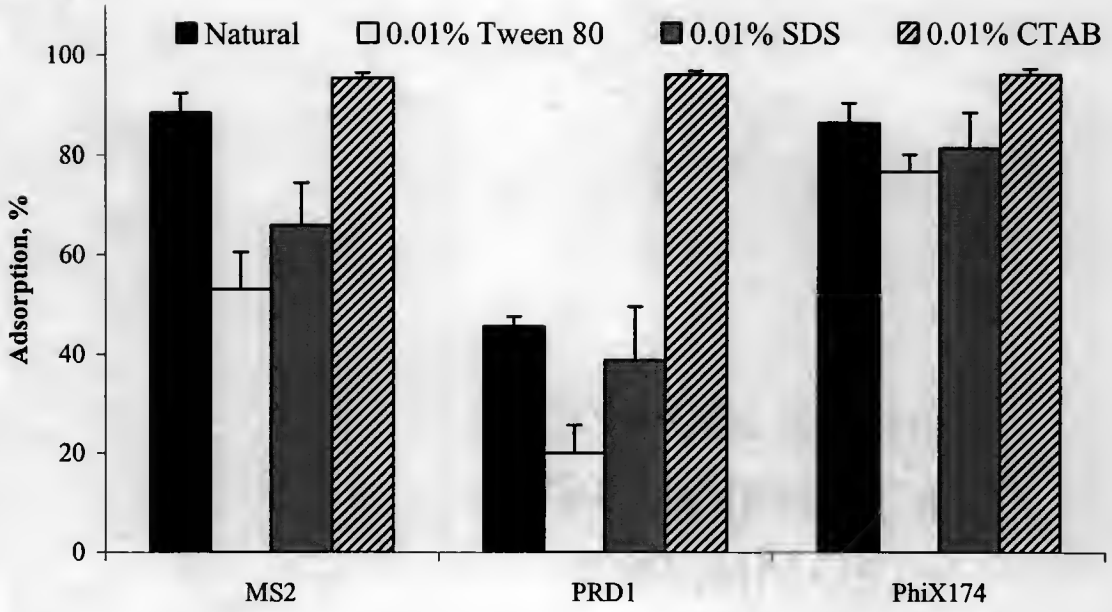

Figure 6-13 Adsorption of viruses in groundwater to soil pretreated with detergents.

Notes: Soil samples (1 g) were mixed for 1 hr with a cationic (hexadecyltrimethylammonium bromide, HTAB), anionic (sodium dodecyl sulfate, SDS), and nonionic (polyoxyethylene sorbitan monooleat, Tween 80) detergent (final concentration 0.01 %) in groundwater. Samples were washed three times with groundwater. Viruses in 2 ml groundwater were mixed with pretreated soils for 1 hr. The sample were centrifuged and the supernatant fraction was assayed to determine percentage of virus adsorption. Each bar is an average and error bars represent standard deviation (n = 3).

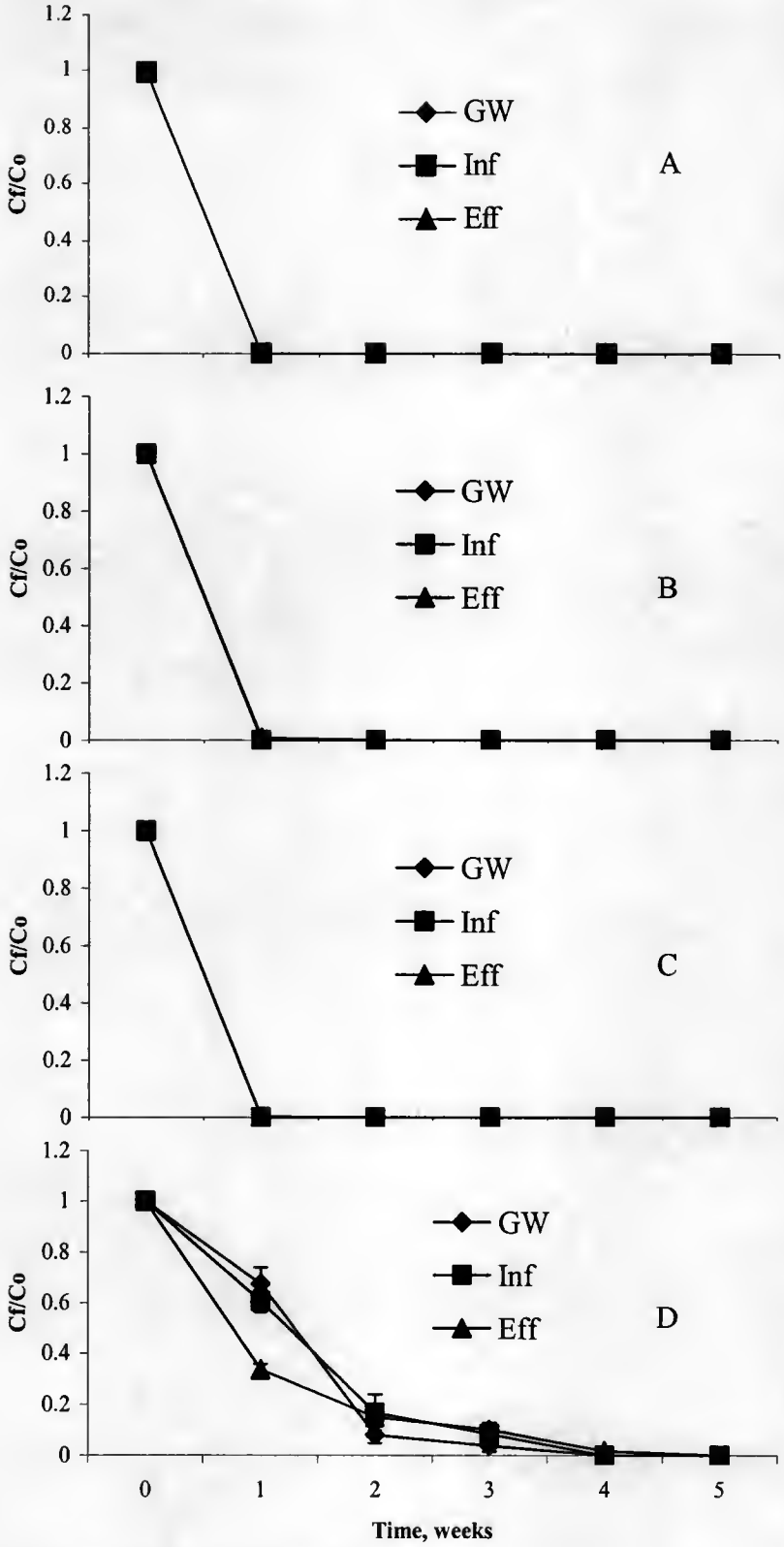

Figure 6-14. Survival of viruses in soil.

Notes: MS2 (A), PRD1 (B), ΦX174 (C), and poliovirus 1 LSc (D) suspended in groundwater (□), influent (□) and effluent (□). Each point is an average and error bars represent standard deviation (n = 3).

CHAPTER 7
SUMMARY OF CONCLUSIONS

Anaerobic digestion has been used for treatment of various wastewaters for energy production, waste stabilization, and pathogen reduction. Anaerobic digestion at mesophilic temperatures and long HRTs is known to reduce the concentrations of pathogenic bacteria from various manures. Decimation during anaerobic digestion is mainly influence by operating temperature and HRT, where higher temperatures and longer HRTs result in higher rates of decimation. In the current study, parameters simulating the operating conditions (i.e., 28°C and 3-day HRT) of the IFAS fixed-film anaerobic digester were used to determine the contribution of other factors to decimation of indicator and pathogenic bacteria.

The current study investigated the influence of the indigenous microflora in manure on the survival of indicator organisms and pathogenic bacteria in the liquid phase during fixed-film anaerobic digestion. The current study found that indicator and pathogenic bacteria were reduced by > 0.8 $\log_{10}$ in the presence indigenous microflora of dairy manure. The anaerobic and facultative population of the indigenous microflora were the active population contributing to decimation.

Limited substrate concentration specifically affected *S. aureus*. In the current study, *S. aureus* was the only organism that could not proliferate in effluent, but was able to grow in influent. This result suggested that: 1) *Staphyolococcus aureus* populations could not be predicted by current indicators and 2) that the organism was limited by the lack of substrate or inhibited in the effluent. Supplementation of effluent with tryptic soy

broth showed that inhibitory compounds were not preventing proliferation of *S. aureus*. This finding suggests that a necessary substrate(s) to support *S. aureus* was removed during anaerobic treatment of influent. Addition of a carbon source, glucose, increased the growth of *S. aureus* in effluent. The inability of *S. aureus* to proliferate in effluent may be due to the lack of sufficient carbon sources, resulting in decimation.

The current results show that the indigenous microflora in manure and the lack of sufficient substrates contributes to the decimation of indigenous indicator organisms and pathogenic bacteria. These findings suggest the mechanisms of decimation of these organisms in the liquid phase during anaerobic treatment. The current study showed reductions of these organisms in the presence of indigenous microflora at ambient temperature (28°C) and a low HRT (3 days). The results of this study demonstrate the importance of indigenous microflora in manure for sanitization of manure wastewater by fixed-film anaerobic digestion. In addition, removal of substrates necessary to support pathogenic bacteria may also play a role in decimation.

Evidence presented in the current study demonstrated the role of the fixed-film in indicator and pathogenic bacteria reduction. The fixed-film may account for removal of indicator and pathogenic bacteria from the liquid phase by attachment to the biofilm. Viability, following attachment, remains to be determined. However, test organisms detected in the effluent were not viable, thus suggesting inactivation in the liquid phase. Earlier work presented in this study demonstrated the role of microbial competition in the liquid phase. Therefore, attachment to the biofilm and microbial competition may work synergistically to remove and inactive indicator and pathogenic organisms during fixed-film anaerobic digestion.

The current study provided evidence demonstrating the adsorption and desorption behavior of selected viruses. Influent was shown to decrease viral adsorption to sandy soil. Viruses present in influent may have the potential to contaminate groundwater during land application. Land application of untreated dairy manure wastewater may increase the movement of viruses through soil, potentially contaminating groundwater.

The use of untreated wastewater may cause mobilization of attached viruses through the soil matrix by interfering with adsorption mechanisms. As demonstrated with MS2, the influent may interfere with hydrophobic and electrostatic interactions between the soil and MS2 due to the increased amount of soluble organics contributed to the soil. Furthermore, influent compounds smaller than 10 kDa may cause inactivation of MS2. However, inactivation was not observed in experiments with the whole fraction of influent. Influent may also interfere with hydrophobic and electrostatic interactions between PRD1 and ΦX174 to soil, where adsorption decreases. Influent compounds smaller than 100 kDa interfered with adsorption of PRD1 and ΦX174 to soil. Anaerobic digestion lowers the soluble organic content and may also remove the fraction interfering with adsorption of PRD1 and ΦX174, resulting in increased virus retention and decreased virus mobilization in the soil.

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BIOGRAPHICAL SKETCH

Johnny A. Davis was born in Gaston, Alabama, on June 3, 1976. In 1978, he moved to Jackson, Mississippi, where he graduated from Murrah High School in 1995. He then attended Florida Agricultural and Mechanical University in Tallahassee, Florida, majoring in biology. He graduated magna cum laude with a Bachelor of Science in May 1999. In the summer of 1999, Johnny began his graduate studies at the University of Florida, under the supervision of Dr. Samuel Farrah. He completed the requirements of a master's degree in December 2001. Since then, he has been fulfilling the requirements for a Doctor of Philosophy degree under the supervision of Dr. Samuel Farrah and Dr. Ann C. Wilkie in the Soil and Water Science Department. After completion of his graduate study, he plans to pursue postdoctoral experiences and eventually a career in academia pursing independent research interests and training the next generation of scientists.